(54) Title: A METHOD OF ASSESSING THE AMOUNT OF NUCLEIC ACID IN A SAMPLE

(57) Abstract: The present invention provides a method of assessing the amount of target nucleic acid in a sample which comprises co-amplifying the target nucleic acid and at least one competitor nucleic acid molecule wherein each competitor molecule contains a unique discriminatory sequence, and determining the relative amounts of the respective amplicons, characterised in that determination is achieved by detecting a primer extension reaction using each said amplicon as template and a kit for use in such a method.

A Method of assessing the amount of nucleic acid in a sample

This invention relates to a method of quantifying nucleic acid and in particular to a competitive assay for nucleic acid which has particular utility in the detection or diagnosis of genetic alterations or infections.

Medical conditions may be diagnosed by the identification of specific target nucleic acid, for example the presence of nucleic acid characteristic of an invading pathogen e.g. viral DNA or RNA and more particularly by monitoring virus copy number. Alternatively, genetic alterations (e.g. mutations) characteristic of or diagnostic for a given medical condition may be detected. Obtaining quantitative data about nucleic acid is useful in analysing the amount of an organism or organisms, e.g. bacteria, in a sample where the quantitative data relating to the nucleic acid is indicative of the number of organisms present in the sample. Also, quantitative information about specific genes, for example by analysis of mRNA, yields useful data about expression patterns of genes of interest. Expression of genes may be up regulated or down regulated in certain medical conditions and such studies are also useful for genomic analyses or environmental monitoring, contamination testing etc.

Target DNA molecules are often present in cell lysates or other source materials in extremely small quantities and in order to amplify such DNA selectively, the polymerase chain reaction (PCR) method has been developed. In this technique a pair of polymerisation primers, specific to known sequences of the target DNA to be amplified, are selected, one primer hybridising at or towards the 5' end of one of the strands of the

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target DNA and the other primer at or towards the 5' end
of the second strand, such that in the presence of a
polymerase, each primer produces a DNA sequence
corresponding to the length of the target DNA template
5   from the terminal of the primer sequence to the other
end of the DNA molecule. If the DNA so produced is then
subjected to strand separation, typically by melting at
a temperature of about 90°C, the newly formed single
stranded DNA sequences will hybridise to excess primer
10   present in the mixture, usually after reducing the
temperature to the range suitable for annealing,
whereupon in the presence of the polymerase, further DNA
strands are synthesised, this time extending only
between the termini of the two primers. The polymerase
15   is preferably capable of surviving the high temperature
used in the strand separation step, thus a thermophilic
polymerase, namely Taq DNA polymerase, may
advantageously be used. If an excess of the two primers
and of nucleotides needed for DNA synthesis is
20   maintained in the medium, it is possible to operate a
repeated cyclic process in which the separate strands
are synthesised, separated, annealed to primer and new
strands synthesised, merely by raising and lowering the
temperature between the optimal temperatures for each of
25   the above stages. In this way, it is found that
amplification of the original target DNA can be
exponential and million-fold increases of concentration
can be effected in a relatively short time.
         In the detection of bacteria, virus and parasites
30   for example, PCR has several advantages compared to
conventional diagnostic methods, i.e. the generality and
speed of the assay. However, the fact that conventional
PCR assays are only qualitative limits their use to
diagnostic applications where only the presence or
35   absence of the pathogen is to be determined. For many
diseases, a quantitative measurement is needed to make a
proper diagnosis and it would be useful to be able to

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measure the amount of pathogen during treatment to make
a relevant prognosis. As mentioned above, there is
major concern regarding the usefulness of PCR assay
because their extreme sensitivity makes it possible to
5   obtain false positives as a result of single molecules
contaminating the sample. Even small differences in the
efficiency of amplification reaction can greatly affect
the final accumulation of PCR products. There is
therefore a need for a quantitative assay, e.g. one
10   suitable for clinical assays, which overcomes the
drawbacks associated with conventional PCR assays.

A number of systems to quantify the initial
template DNA/RNA have been described (see for example A.
C. Syvänen, M. Bengtström, J. Tenhunen and H. Söderlund
15   Nucl. Acids Res., 16, 11327 (1988); G. Gilliland, S.
Perrin and H. F. Bunn PCR protocols, pp. 60-69, Academic
Press, San Diego (1990); M. Becker-André and K.
Hahlbrock, Nucl. Acids Res. 17,9437 (1990); and N. Kato,
O. Yokosuka, K. Hosoda, Y. Ito, M. Okto and M. Omata,
20   Hepatology 18, 16-20 (1993)).

Such methods involve quantization techniques e.g.
based on isotope labelling or restriction analysis,
which are difficult or cumbersome to operate and time
consuming to perform. Such methods are not therefore
25   readily amenable to automation or to the analysis of
large numbers of different samples.

A competitive PCR-based quantitation technique
described by Cemu Bioteknik AB in WO 92/01812 represents
an improvement over such techniques, but nonetheless has
30   drawbacks when it comes to the analysis of a large
number of samples, for example in a diagnostic
situation. This method is based on the technique
described in WO 90/11369 for the detection of
immobilized amplified nucleic acids (designated DIANA)
35   and involves competitive titration wherein amounts of
target DNA are co-amplified with differing, known
amounts of competitor DNA to produce different ratios of

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target: competitor DNA, the competitor DNA being
substantially the same as the target DNA except that is
comprises a recognition site which may be detected
directly or indirectly by a labelled species. Different
5   known amounts of the competitor DNA are added to
aliquots of the sample generally as a series of equally
stepped dilutions. A set of readings corresponding to
the label values in each aliquot is obtained which, when
plotted against the known amounts of added competitor
10 DNA give a characteristic sigmoid curve; the point of
inflection on the curve is defined by the sharp change
in the amount of labelled DNA between those aliquots in
which added competitor DNA predominated and those in
which target DNA predominated and is approximately
15 proportional to the amount of target DNA in the initial
sample.

        Whilst the so-called "quantitative DIANA" technique
of WO 92/01812 avoids the use of disadvantageous
quantification methods inherent in the other prior art
20 methods mentioned above, it requires that a large number
of determinations (and hence PCR reactions) be
performed, in order to determine one unknown. Thus for
example, for one sample at least 8 different measurement
points are in practice required, in the form of a
25 dilution series of the competitor DNA. The technique is
therefore not only costly, in terms of the large number
of reactions required, but requires large initial sample
volumes. This renders the method unsuited to the
analysis of large numbers of samples, such as occurs in
30 a clinical diagnostic laboratory.

        Various other competitive PCR techniques used to
generate quantitative data are known. Competitive PCR
is based on the addition to the sample of a known amount
of a competitor molecule (typically DNA or RNA) which
35 acts as an internal standard. This competitor molecule
has the same primer recognition sites as the target gene
and thus is expected to behave in the same way during

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PCR cycling.

The next step is the method of discrimination
between target and competitor and the technique used to
obtain information regarding how much of each molecule
5   is present after amplification. In competitive PCR
(where the amount of competitor in absolute terms is
known) the absolute determination of the signals
corresponding to the target and the competitor is not
required. The final quantitation of the target is
10   simply derived from the ratio between target and
competitor. A common method for discrimination between
target and competitor is through generation of amplicons
which differ in length. Separation is thus achieved by
gel-electrophoresis and ethidium bromide staining of the
15   bands. Radioactively or fluorescence-labelled dNTPs can
provide a more sensitive procedure than ethidium bromide
staining. However amplicon length based discrimination
is not ideal as the length of the template molecule may
affect amplification efficiency.
20   A PCR product detection method taking advantage of
the 5' exonuclease activity of Taq DNA polymerase and
dual-labelled fluorescent probes has also been described
(Heid, C.A. et al. [1996] Genome Research 6, 986-94).
In the event of successful amplification, the detection
25   probe hybridises to the target/competitor and thereby it
is accessible to the 5'→3' exonucleases activity of the
Taq DNA polymerase. The detection probe is non-
extendable and incorporates a fluorescent reporter dye
and a quencher dye. Due to the closeness of quencher to
30   reporter, the reporter fluorescence is suppressed until
cleavage of the probe occurs during PCR cycling. This
is a rather expensive technique and there is a need for
a more cost-effective and technically simple assay.

It has now been found that a simple and reliable
35   method for obtaining qualitative and quantitative data
about nucleic acid in a sample, which allows high
throughput, can be performed using an internal standard

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where discrimination is achieved by detection of a
primer (chain) extension reaction. Whether or not an
extension reaction takes place on a given template will
be dependent upon the normal rules of base pairing,

5   either at the extension stage and/or at extension primer
hybridisation. The method disclosed herein is
particularly suited to automation e.g. in systems where
the washing and injection steps take place in a
microtitre plate format. The methods are particularly

10   suitable for monitoring virus copy number.

Accordingly, the present invention provides a
method of assessing the amount of target nucleic acid in
a sample which comprises co-amplifying the target
nucleic acid and at least one competitor nucleic acid

15   molecule wherein each competitor molecule contains a
unique discriminatory sequence, and determining the
relative amounts of the respective amplicons,
characterised in that determination is achieved by
detecting a primer extension reaction using each said

20   amplicon as template.

Analysis of the data obtained by the above method
may be performed immediately or at a later time by the
person performing the above determination or by others.
Thus, according to the invention is also provided a

25   method of assessing the amount of target nucleic acid in
a sample which comprises co-amplifying the target
nucleic acid and at least one competitor nucleic acid
molecule wherein each competitor molecule contains a
unique discriminatory sequence, and determining the

30   relative amounts of the respective amplicons, wherein
the information about the relative amounts of the
respective amplicons is correlated to provide a value
for the amount of target nucleic acid in the sample,
characterised in that determination is achieved by

35   detecting a primer extension reaction using each said
amplicon as template.

Conveniently, the target nucleic acid may be DNA,

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although quantitation of target RNA (e.g. mRNA) is also
within the scope of the invention. If it is desired to
assess the amount of RNA in a sample, the method will
additionally include the step of generating cDNA from

5   RNA prior to co-amplification. Such synthesis can be
carried out in an initial treatment step with a reverse
transcriptase, conveniently in the same system of
buffers and bases to be used in the subsequent
amplification. Since the amplification procedures

10   require heating to effect strand separation, the reverse
transcriptase may be inactivated in the first
amplification cycle. The enzymatic activity of both a
reverse transcriptase and a polymerase which is stable
may thus be used which conveniently may take the form of

15   an enzyme with both activities, for example the
polymerase. When mRNA is the target nucleic acid, it
may be advantageous to submit the initial sample, e.g. a
serum sample, to treatment with an immobilized poly dT
oligonucleotide in order to retrieve all mRNA via the

20   terminal poly A sequences thereof. Alternatively, a
specific oligonucleotide sequence may be used to
retrieve the RNA via a specific RNA sequence. The
oligonucleotide can then serve as a primer for cDNA
synthesis, as described in WO 90/06044.

25   It will be appreciated that whilst the target RNA
may first be reverse transcribed into cDNA prior to
amplification in the presence of one or more competitor
DNA molecules, more conveniently, one or more competitor
RNA molecules may be introduced at the stage of reverse

30   transcription such that cDNAs of the target and
competitor RNA are produced which may then be amplified.
The sample may be any sample containing genetic
material, and all biological and clinical samples are
included i.e. any cell or tissue sample of an organism,

35   or any body fluid or preparation derived therefrom, as
well as samples such as cell cultures, cell
preparations, cell lysates etc. Environmental samples,

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e.g. soil and water samples or food samples are also
included. The samples may be freshly prepared or they
may be prior-treated in any convenient way e.g. for
storage. The target nucleic acid may thus be derived or
obtained from all such sources of nucleic acid material.

Representative samples thus include any material
containing nucleic acid, including for example foods and
allied products, clinical and environmental samples.
However, the sample will generally be a biological
sample, which may contain any viral or cellular
material, including all prokaryotic or eukaryotic cells,
viruses, bacteriophages, mycoplasmas, protoplasts and
organelles. Such biological material may thus comprise
all types of mammalian and non-mammalian animal cells,
plant cells, algae including blue-green algae, fungi,
bacteria, protozoa etc. Representative samples thus
include whole blood and blood-derived products such as
plasma, serum and buffy coat, urine, faeces,
cerebrospinal fluid or any other body fluids, tissues,
cell cultures, cell suspensions etc. The sample may be
pre-treated in any convenient or desired way to prepare
the nucleic acid molecule for use (i.e. reaction) in the
method of the invention, for example by cell lysis or
purification, isolation, copying or pre-amplification of
the nucleic acid etc.

The term "co-amplifying" includes any method for *in
vitro* amplification which can be used to amplify both
the target nucleic acid and competitor sequences. In
this regard, both linear and exponential amplification
methods may be used. As will be described further
below, any convenient or desired method of *in vitro*
amplification may be used, according to techniques well
known and described in the art. The competitor
molecules may conveniently be added to the sample, or to
aliquots of the sample. Conveniently, the target and
competitor molecules are amplified using the same
amplification primers.

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The term "unique discriminatory sequence" refers to a region of the competitor nucleic acid molecule which differs both from the equivalent region in the target molecule and the equivalent region in any other

5   competitor molecule present in the amplification reaction. Thus, each competitor will comprise a region of sequence variation, wherein this region (i.e. the "sequence") is different from the target and different from other competitors. The "sequence" comprises at

10   least one base, preferably more than one, more preferably more than two bases. Suitable sequences for use in the methods of the invention comprise 2-10 bases, typically 3-10, 3-8 or 3-6, preferably 3-5, more preferably 3 or 4 bases. Thus, the unique

15   discriminatory sequence may be the above ranges in length i.e. 1 to 10 bases, or 3-10, 3-8, 3-6, 3-5, 3 or 4 bases etc.

     Preferably the discriminatory sequence will be a homopolymer, e.g. 2 or more or 3 or more, e.g. 3-6, 2-6,

20   or more preferably 3 or 4 or 5 adjacent identical bases. The discriminatory sequences will typically be predetermined and the competitor molecule will have been designed to incorporate the unique discriminatory sequence but preferably be substantially the same as or

25   identical to the target sequence and any other competitor molecule apart from in the region of the discriminatory sequence. The competitor molecule may conveniently be linear or added to the sample as a plasmid.

30   The amplicons derived from the target and competitor nucleic acid molecules will conveniently incorporate the unique discriminatory sequence or the equivalent region in the target molecule. Apart from the discriminatory sequence, the amplicons derived from

35   the competitor molecule will be substantially the same as the target molecule and any other competitor molecule present. The amplicons will be substantially the same

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apart from the unique discriminatory sequence. By
'substantially the same' is meant the sequences have at
least a 70%, preferably at least a 75% or 80% more
preferably at least a 90, 95 or 98% sequence identity,
as determined using the Fasta search (Pearson and Lipman
(1988), Proc. Natl. Acad. Sci. USA 5: 2444-2448) as part
of the GCG packages using default values; word size: 6;
Gap creation penalty: 12.00; Gap extension penalty: 4.0,
and constant Pam factor. The amplicons can also be
regarded as 'substantially the same' if the amplicon
derived from the competitor molecule is capable of
hybridising to the complement of the amplicon derived
from the target molecule under high stringency
conditions. Conditions of high stringency may readily
be determined according to techniques well known in the
art, as described for example in Sambrook et al., 1989,
Molecular Cloning, A Laboratory Manual, 2nd Edition.
Hybridising sequences included within the scope of the
invention are those binding under non-stringent
conditions (6 x SC/50% formamide at room temperature)
and washed under conditions of high stringency (e.g. 2 x
SSC, 65°C), where SSC = 0.15 M NaCl, 0.015M sodium
citrate, pH 7.2. Generally speaking, the aim is for the
target and competitors to be as similar as possible,
(aside from the discriminatory region) and consequently,
it is preferred for variation between the target and
competitors to be minimised outside the discriminatory
region, and in particular for the variation to be
confined to the discriminatory region. Thus, it is
preferred for the target and competitor to be
substantially similar in the region which is to be
amplified. In other words, it is preferred that the
competitor and target molecules are identical in
sequence, except in the discriminatory region. However,
it must be accepted that minor variation may occur, for
example due to base misincorporation etc.

The amplicons derived from competitor and target

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molecules are preferably substantially the same length,
i.e. differ in length by less than 30 bases, preferably
less than 20 or 10 bases, most preferably are identical
in length or are within 2 or 3 bases of the same length.
5   Similarity of amplicons derived from the competitor
molecules with each other and with the target in terms
of length and C/G base composition is more important
than similarity between the original starting competitor
molecule and the target molecule.

10   Typically, before the method of the invention is
performed a region of the target molecule will be
identified, preferably one which provides 2 or more,
e.g. 3 or 4 continuous identical bases. However the
"region" may comprise just a single base. A competitor
15   molecule will then be designed which is substantially
the same as the target molecule (e.g. in the region to
be amplified) except for the aforementioned identified
region which in the competitor molecule provides the
unique discriminatory sequence. The identified region
20   in the target molecule will therefore be equivalent to
the unique discriminatory sequence in the competitor and
is referred to as such herein.

       Preferably, the competitor molecule contains a
homopolymeric discriminatory sequence which is
25   conveniently the same length as the homopolymeric region
identified in the target but is made up of a different
base. Further competitor molecules for use in the same
method may be provided in an analogous way,
incorporating a homopolymeric discriminatory sequence
30   made up of a base other than those found in the
identified region of the target molecule or any other
competitor molecules. Thus, as nucleic acid typically
comprises 4 different bases, there will advantageously
be a target molecule and three types of competitor
35   molecule, each type of competitor having a homopolymeric
region made up of a different base.

       In order to perform a primer extension reaction, it

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will be necessary to provide a primer for hybridisation
to the amplicons. This "extension" primer may be one of
the primers used in the amplification reaction but will
preferably be a different primer. According to
5   different versions of the method of the invention, the
extension primer may be 'generic', i.e. the same primer
is able to bind to the target nucleic acid and any
amplicon derived from a competitor molecule. Such a
generic primer may be one of the amplification primers.
10   Advantageously, such a primer will bind substantially
adjacent (e.g. within 6, preferably within 3 bases),
preferably exactly adjacent to the unique discriminatory
sequence of the competitor and the equivalent region of
the target molecule. A primer extension reaction may
15   then be performed dependent upon availability of
nucleotides which can form a sequence which is
complementary to the discriminatory sequence or the
equivalent region in the target molecule.

Alternatively, different extension primers are
20   provided, each being specific for either the target or a
competitor molecule. Thus, these primers have a region
which is complementary either to one of the unique
discriminatory sequences of a competitor molecule or to
the equivalent region in the target molecule. The
25   specificity is of course achieved by virtue of
complementary base pairing and, for all embodiments of
the invention, primer design may be based on principles
well known in the art. It is not necessary for the
primer to have absolute complementarity to the
30   discriminatory sequence or the equivalent region in the
target molecule, but this may be preferred to improve
specificity of binding. Such primers may be termed
"match/mismatch primers", wherein specificity for one of
the substrates (a competitor or target molecule) means
35   mismatch for the other substrate(s). The mismatch
sequence is preferably located at the 3' end of the
primer. In this embodiment of the invention, primer

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extension does not depend on the sequences adjacent to
the primer but on the capability of an exonuclease-
deficient DNA polymerase to distinguish between a match
and a mismatch (at the 3' termini) primer in an
5   extension assay.

The 'primer extension reaction' according to the
invention includes all forms of template-directed,
polymerase-catalysed nucleic acid synthesis reactions.
The primer extension reaction may only comprise the
10   incorporation of one nucleotide, or may comprise the
incorporation of 2, 3 or more nucleotides.

It will be understood that absolute specificity of
primer-binding and extension cannot be guaranteed, and
some tolerance must be allowed for, as in any biological
15   system. Thus, references herein to specificity are
intended to refer to substantial specificity e.g. of
primer binding, such that the invention may nonetheless
be performed.

Specificity of primer binding and extension may be
20   improved by appropriate primer design and/or selection
of conditions e.g. by designing primers with higher
annealing temperatures to enable the primer annealing
step to be carried out at higher more stringent
temperatures.

25   Alternatively or additionally, the primer extension
reaction may be performed at higher temperatures using a
temperature-tolerant polymerase enzyme.

Conditions and reagents for primer extension
reactions are well known in the art, and any of the
30   standard methods and enzymes etc. may be used in this
step (see e.g. Sambrook et al, (editors), Molecular
Cloning: a laboratory manual (1989), Cold Spring Harbor
Laboratory Press). Thus, the primer extension reaction
is carried out in the presence of primer,
35   deoxynucleotides and a suitable polymerase enzyme e.g.
T7 polymerase, Klenow or Sequenase Ver 2.0 (USB USA), or
indeed any suitable available polymerase enzyme.

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Conditions may be selected according to choice, having
regard to procedures well known in the art.

     According to the present invention, detection of a
primer extension reaction can be performed in a number
5   of ways, such as incorporation of labelled nucleotides
(i.e. by detecting incorporation of a labelled
nucleotide into the primer extension product, for
example by detecting the amount of label in the primer
extension reaction product) or by using labelled probes
10   which are able to bind to the extended sequence.
Alternatively a "TaqMan" probe may be used, which is a
composite probe containing a reporter dye and quencher
dye. (see Heid et al., supra). The additional TaqMan
probe is annealed to the substrate (ssDNA) and extension
15   may be monitored by the exonuclease activity of the
polymerase, which degrades the TaqMan probe.

     Preferably however, primer extension is detected by
monitoring PPi release, preferably by luminometric
detection.

20   PPi can be determined by many different methods and
a number of enzymatic methods have been described in the
literature (Reeves et al., (1969), Anal. Biochem., 28,
282-287; Guillory et al., (1971), Anal. Biochem., 39,
170-180; Johnson et al., (1968), Anal. Biochem., 15,
25   273; Cook et al., (1978), Anal. Biochem. 91, 557-565;
and Drake et al., (1979), Anal. Biochem. 94, 117-120).

     It is preferred to use luciferase and luciferin in
combination to identify the release of pyrophosphate
since the amount of light generated is substantially
30   proportional to the amount of pyrophosphate released
which, in turn, is directly proportional to the amount
of base incorporated. The amount of light can readily
be estimated by a suitable light sensitive device such
as a luminometer.

35   Luciferin-luciferase reactions to detect the
release of PPi are well known in the art. In
particular, a method for continuous monitoring of PPi

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release based on the enzymes ATP sulphurylase and
luciferase has been developed (Nyrén and Lundin, Anal.
Biochem., 151, 504-509, 1985; Nyrén P., Enzymatic method
for continuous monitoring of DNA polymerase activity
5   (1987) Anal. Biochem Vol 167 (235-238)) and termed ELIDA
(Enzymatic Luminometric Inorganic Pyrophosphate
Detection Assay). The use of the enzymes luciferase and
ATP sulphurylase, and particularly the use of the ELIDA
method to detect PPi is preferred according to the
10   present invention. The method may however be modified,
for example by the use of a more thermostable luciferase
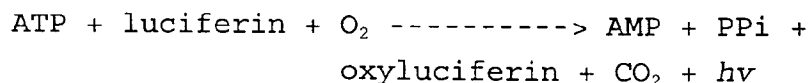(Kaliyama et al., 1994, Biosci. Biotech. Biochem., 58,
1170-1171) and/or ATP sulfurylase (Onda et al., 1996,
Bioscience, Biotechnology and Biochemistry, 60:10, 1740-
15   42). This method is based on the following reactions:

                ATP sulphurylase
    PPi + APS ---------------> ATP + SO$_4^{2-}$

20   luciferase
    ATP + luciferin + O$_2$ ----------> AMP + PPi +
                    oxyluciferin + CO$_2$ + hv
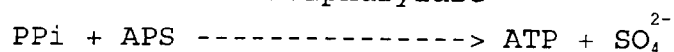
    (APS = adenosine 5'-phosphosulphate)
25

Reference may also be made to WO 98/13523 and WO
98/28448, which, whilst directed to pyrophosphate
detection-based sequencing procedures, disclose PPi
detection methods which may be of use in the present
30   invention.
     A potential problem which has previously been
observed with PPi-based sequencing methods is that dATP,
used in the chain extension reaction, (or any other
adenine nucleotide used in the primer extension reaction
35   such as dideoxy ATP (ddATP)) interferes in the
subsequent luciferase-based detection reaction by acting
as a substrate for the luciferase enzyme. This may be

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reduced or avoided by using, in place of deoxyadenosine
triphosphate (ATP) (i.e. in place of the adenine
nucleotide), a dATP (or other adenine nucleotide)
analogue which is capable of acting as a substrate for a
5   polymerase but incapable of acting as a substrate for a
PPi-detection enzyme.

The term "incapable of acting" includes also
analogues which are poor substrates for the detection
enzymes, or which are substantially incapable of acting
10   as substrates, such that there is substantially no,
negligible, or no significant interference in the PPi
detection reaction.

Thus, a further preferred feature of the invention
is the use of a dATP analogue which does not interfere
15   in the enzymatic PPi detection reaction but which
nonetheless may be normally incorporated into a growing
DNA chain by a polymerase. By "normally incorporated"
is meant that the nucleotide is incorporated with
normal, proper base pairing. In the preferred
20   embodiment of the invention where luciferase is a PPi
detection enzyme, the preferred analogue for use
according to the invention is an α-thio analogue, e.g.
the [1-thio]triphosphate (or α-thiotriphosphate)
analogue of deoxy ATP, preferably deoxyadenosine [1-
25   thio]triphospate, or deoxyadenosine α-thiotriphosphate
(dATPαS) as it is also known. dATPαS, along with the α-
thio analogues of dCTP, dGTP and dTTP, may be purchased
from New England Nuclear Labs. Experiments have shown
that substituting dATP with dATPαS allows efficient
30   incorporation by the polymerase with a low background
signal due to the absence of an interaction between
dATPαS and luciferase. The signal-to-noise ratio is
increased by using a nucleotide analogue in place of
dATP, which eliminates the background caused by the
35   ability of dATP to function as a substrate for
luciferase. In particular, an efficient incorporation
with the polymerase may be achieved while the background

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signal due to the generation of light by the luciferin-
luciferase system resulting from dATP interference is
substantially decreased. The α-thio analogues of other
nucleotides (e.g. dNTPαS analogues of the other
5   nucleotides) may also be used in place of all
nucleotides (e.g. dNTPs).

       The pyrophosphate detection step results in a
signal indicative of the amount of pyrophosphate
released. It has been confirmed that this signal is
10   proportional to the amount of template present i.e.
amplicons derived from target or competitor and
therefore the signal is proportional to the number of
target and competitor molecules originally present. If
the amount of competitor is known, it follows that the
15   amount of target can be calculated. Preferably, a
calibration curve based on detection of extension
reactions from competitor templates is generated and
this can be used to read off quantitative information
regarding the amount of target nucleic acid present in
20   the sample. There is a linear correlation between the
signal (i.e. the amount of PPi released) and the
relative amount of template.

       The term "target nucleic acid" is intended to
encompass _inter alia_ DNA, cDNA e.g. from retroviral RNA,
25   genomic DNA, mitochondrial DNA, RNA, mRNA and PNA. The
DNA may be single or double stranded.

       The term "complementary" as used herein is intended
to encompass any nucleic acid molecule which is
complementary to the nucleic acid in question, its
30   complementary sequence or its RNA or DNA equivalent or
complementary sequence thereof. Whilst absolute
complementarity is preferred, less than absolute
complementarity, for example due to the occasional base
misincorporation may be tolerated. In general, it is
35   accepted for the complementarity to be "substantial",
within the limits and definitions given above in
relation to "substantial identity" i.e. with the %

homology and hybridisation conditions and limits given above.

The term "competitor nucleic acid" as used herein is intended to encompass any piece of DNA (or RNA after reverse transcription or PNA) which would compete with the target DNA (or RNA after reverse transcription or PNA) for binding to at least one of the primers used in the amplification reaction. "Competitor nucleic acid" extends also to the use of chimers of RNA, DNA and/or PNA. It will be appreciated however that it is an essential requirement of the method that all the nucleic acid sequences which are amplified in the amplification reaction must be amplified at a comparable rate and must therefore not be restricted with regard to the availability of essential reagents, ie. primers. Thus although referred to as competitor nucleic acid, this nucleic acid would only compete with the target nucleic acid when limiting concentrations of primers were available. For performance of the invention, this would not be the case. An excess of primers is generally used. The competitor may be single or double stranded.

The term "assessing" as used herein includes both quantitation in the sense of obtaining an absolute value for the amount of target nucleic acid in a sample, and also obtaining a semi-quantitative assessment or other indication, eg an index or ratio, of the amount of target nucleic acid in the sample. Thus, for example a ratio of the amount of target to competitor molecule may be obtained. The competitor molecule(s) is(are) subjected to the co-amplification step in a known amount (e.g. a known amount or concentration of the competitor may be added to the target nucleic acid or to the sample). Since the amount of competitor is known, it may be used to assess the amount of target.

More particularly, "assessing" is used herein to refer to obtaining a quantitative assessment of the amount of target nucleic acid in the sample by virtue of

a comparison of the amount of target nucleic acid
present in a sample with a known amount of a competitor.
Such a comparison is allowed due to measurement of a
primer extension reaction where both target and

5   competitor provide/act as templates. As discussed
herein, the invention provides methods whereby extension
of a primer hybridised to an amplicon derived from a
target molecule can be differentiated from extension of
a primer hybridised to an amplicon derived from a

10   competitor molecule. Differentiation may be achieved
through specificity of extension primer hybridisation or
through the extension reaction itself, for example by
the ability to bring about an extension reaction when
nucleotide availability is restricted. For example,

15   there will only be a primer extension reaction when the
extension primer is annealed adjacent to the
discriminatory sequence if a nucleotide for
incorporation is supplied which forms a base pair with
the nucleotide adjacent to the 3' end of the extension

20   primer.

As mentioned above, the method of the invention may
comprise the step of correlating the relative amounts of
the respective amplicons (i.e. as obtained in the co-
amplification step) to determine, or assess, the amount

25   of target nucleic acid in the sample. As discussed
above, the "relative amounts" may be an absolute
assessment of the amounts of the respective amplicons
(and hence of the respective amounts of the target and
competitor molecules present (e.g. in the sample)).

30   Alternatively, a ratio of the respective amounts may be
obtained, or some other index or indication which may
provide an index, or indication, or value for the amount
of target nucleic acid in the sample. The "correlation"
step may be performed simply by comparing the respective

35   amounts determined or assessed, or by any other
technique or calculation known in the art, for example
by plotting a curve, comparing with a standard curve

etc.

As mentioned above, the method requires that the competitor and target nucleic acids are amplified to the same extent, i.e. substantially at the same rate or with the same or substantially the same amplification efficiency, albeit to different levels as a result of the presence of different concentrations in the starting reaction. It will thus be appreciated that it is advantageous to ensure that the size and GC-content of the nucleic acids are kept substantially the same and that the sequence/s where the primer/s binds is identical. For this reason it is preferable to use competitor nucleic acids which have substantially the same sequence as the target nucleic acid; excluding the unique discriminatory region as discussed above.

Optionally, the competitor nucleic acid may be provided with a means for immobilization, which may be introduced during amplification, either through the nucleotide bases or the primer/s which is used to produce the amplified nucleic acid. The target nucleic acid may similarly be provided with a means for immobilization as a result of the amplification procedure. Preferably, therefore, one of the amplification primers will carry means for immobilization, or may be provided already immobilised on a solid support. Thus, the respective amplicons obtained may carry, or be provided with means for immobilisation or may be immobilised.

To facilitate immobilization, the primers used according to the invention may carry a means for immobilization either directly or indirectly. Thus, for example the primers may carry sequences which are complementary to sequences which can be attached directly or indirectly to an immobilizing support or may carry a moiety suitable for direct or indirect attachment to an immobilizing support through a binding partner.

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Numerous suitable supports for immobilization of
DNA and methods of attaching nucleotides to them, are
well known in the art and widely described in the
literature. Thus for example, supports in the form of
5   microtitre wells, tubes, dipsticks, particles, fibres or
capillaries may be used, made for example of agarose,
cellulose, alginate, teflon, latex or polystyrene.
Advantageously, the support may comprise magnetic
particles eg. the superparamagnetic beads produced by
10   Dynal AS (Oslo, Norway) and sold under the trademark
DYNABEADS. Chips may be used as solid supports to
provide miniature experimental systems as described for
example in Nilsson et al. (Anal. Biochem. (1995),
224:400-408).

15   The solid support may carry functional groups such
as hydroxyl, carboxyl, aldehyde or amino groups for the
attachment of the primer or capture oligonucleotide.
These may in general be provided by treating the support
to provide a surface coating of a polymer carrying one
20   of such functional groups, eg. polyurethane together
with a polyglycol to provide hydroxyl groups, or a
cellulose derivative to provide hydroxyl groups, a
polymer or copolymer of acrylic acid or methacrylic acid
to provide carboxyl groups or an amino alkylated polymer
25   to provide amino groups. US patent No. 4,654,267
describes the introduction of many such surface
coatings.

Alternatively, the support may carry other moieties
for attachment, such as avidin or streptavidin (binding
30   to biotin on the nucleotide sequence), DNA binding
proteins (eg. the lac I repressor protein binding to a
lac operator sequence which may be present in the primer
or oligonucleotide), or antibodies or antibody fragments
(binding to haptens eg. digoxigenin on the nucleotide
35   sequence). The streptavidin/biotin binding system is
very commonly used in molecular biology, due to the
relative ease with which biotin can be incorporated

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within nucleotide sequences, and indeed the commercial
availability of biotin-labelled nucleotides this
represents one preferred method for attachment of.
Streptavidin-coated DYNABEADS are commercially available
5   from Dynal AS.

    As mentioned above, immobilization may conveniently
take place after amplification. To facilitate post
amplification immobilisation, one or both of the
amplification primers are provided with means for
10   immobilization. Such means may comprise as discussed
above, one of a pair of binding partners, which binds to
the corresponding binding partner carried on the
support. Suitable means for immobilization thus include
biotin, haptens, or DNA sequences (such as the lac
15   operator) binding to DNA binding proteins.

    When immobilization of the amplification products
is not performed, the products of the amplification
reaction may simply be separated by for example, taking
them up in a formamide solution (denaturing solution)
20   and separating the products, for example by
electrophoresis or by analysis using chip technology
(mentioned hereinafter). Immobilization provides a
ready and simple way to generate a single-stranded
template for the extension reaction. As an alternative
25   to immobilization, other methods may be used, for
example asymmetric PCR, exonuclease protocols or quick
denaturation/annealing protocols on double stranded
templates may be used to generate single stranded DNA.
Such techniques are well known in the art.

30   The method of the invention may be performed in a
number of different ways. A number of particular
specific embodiments are described further below, but
generally speaking, the method may be performed using
multiple competitor molecules or a single competitor
35   molecule, and/or different amounts of the competitor
molecules. Different primer extension reactions may be
performed, (using the same or different extension

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primers, as mentioned above).

The principle is to perform a number of different primer extension reactions based on the competitor derived template(s), which may be selectively or

5   specifically detected to generate signals (or calibration points) which may be correlated to the amount of template for each competitor or each primer extension reaction, and thus provide a basis for assessment of the amount of target nucleic acid in the

10   sample (e.g. by comparison with the primer extension reaction(s) on a target-derived amplicon). Such a multiplicity of primer extension reactions may be performed by using a multiplicity of different competitor molecules, each used or present in a

15   different amount, and performing a primer extension reaction on each different competitor amplicon, e.g. using either the same primer (and a template-specific or template-directed primer extension reaction, which as mentioned above may be accomplished by restricting the

20   availability of nucleotides for incorporation or by using particular nucleotides) or by using different, template-specific extension primers, in order to discriminate or differentiate between the various competitor and target-based extension reactions.

25   Alternatively, a single competitor may be used, but a multiplicity of different primer extension reactions may be performed, for example to generate primer extension products which may be distinguished, or to provide distinguishable primer extension signals, e.g. as

30   discussed further below.

As used herein the terms "multiple" or "multiplicity" refer to 2 or more, e.g. 3 or more or 4, 5, 6 or more etc. e.g. 2 to 10, or more particularly 2 to 6, 2 to 5, 2 to 4, and preferably 3 or 4.

35   Where more than one competitor molecule is used, the concentrations of competitor nucleic acid should be selected to provide a standard curve (i.e. a calibration

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curve) in which the concentration of target nucleic acid
falls within the range of the lowest and highest
concentration of the competitor nucleic acid.

Thus, for example, competitors at a concentration (or
5   copy number) of 10x, 100x and 1000x would allow the
determination of target nucleic acid at a concentration
(or copy number) of 10x to 1000x. This range of
concentrations can be used in low-copy applications such
as HIV-1 using nested PCR or when the target is present
10   at a high copy number/concentration such as expression
analysis in which a single PCR run is sufficient to
assess variations in expression levels.

For the preparation of an internal calibration
curve, at least two, preferably three different nucleic
15   acid competitors at different concentrations should be
used in the assay, unless the method shown in Fig. 3 is
performed, which requires only one competitor
concentration.

It will be appreciated that the sequence and length
20   of the oligonucleotides to be used as amplification
primers according to the invention will depend on the
sequence of the target nucleic acid, the desired length
of amplification product, the further functions of the
primer (eg. means for immobilization) as well as the
25   amplification procedure.

The in vitro amplification reaction may be any
process which amplifies the nucleic acid present in the
reaction under the direction of appropriate primers.
The method may thus preferably be performed by PCR, and

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the various modifications thereof e.g. the use of nested
primers, although it is not limited to this method. PCR
will however generally be the method of choice. Those
skilled in the art will appreciate that the invention

5   would also be appropriate with amplification procedures
such as Self-sustained Sequence Replication (3SR),
NASBA, the Q-beta replicase amplification system and
Ligase chain reaction (LCR) (see for example Abramson
and Myers (1993) Current Opinion in Biotech., 4: 41-47).

10   Advantageously, to minimise any possible
differences due to amplification efficiency, sufficient
cycles of amplification are performed, to reach a
saturation level (the so-called "plateau" phase of
amplification). However, this is not essential and

15   amplification may be performed in the exponential phase.
If necessary, appropriate controls may be used to
compensate for any differences in amplification,
efficiency etc. The use of such controls is routine and
widely known in the field of in vitro amplification.

20   For the determination of the number of copies (or
concentration or amount) of target nucleic acid in the
sample, the signals derived from primer extension of the
amplified competitor nucleic acid may be used to
generate a standard curve in which the level of signal

25   is plotted against copy number (or concentration or
amount) prior to amplification. Once the standard curve
has been generated this can be used to read off the
amount of starting copies (or concentration or amount)
of target nucleic acid from the sample as reflected by

30   the level of signal generated by primer extension of the
amplified target nucleic acid. The "signal" may be
luminescence when release of PPi is monitored but may
derive from an incorporated label, e.g. a fluorescent or
radio-label.

35   This method thus not only qualitatively positively
identifies the presence of target sequence, unlike a
number of the assays based on competition between target

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and competitor nucleic acid sequences for primers, but also allows the quantification of the amount of target nucleic acid present in the sample. Thus, the present invention provides a convenient single tube

5   amplification protocol which allows quantification of PCR amplicons in a non-gel based bioluminometric assay.

Furthermore, by relying on a standard curve the method of the invention thus avoids the need for determination of the actual amount of amplified target

10 DNA although this may in some cases be useful information.

Two-stage PCR (using nested primers), as described in WO90/11369, may be used to enhance the signal to noise ratio and thereby increase the sensitivity of the

15 method according to the invention.

Regardless of whether one-stage or two stage PCR is performed, the efficiency of the PCR is not critical since the invention relies on amplification of competitor and target nucleic acid in the same reaction

20 and thus all nucleic acid is amplified to the same extent.

The quantitative method according to the invention may be used for general quantification of RNA and DNA both for research and clinical applications, including

25 diagnosis of viral, bacterial and protozoan pathogens. It may also find applications in forensic medicine, or environmental or contamination testing or monitoring.

Any suitable polymerase may be used, although it is preferred to use a thermophilic enzyme, such as Taq DNA

30 polymerase, to permit the repeated temperature cycling without having to add further polymerase, e.g. Klenow fragment, in each cycle.

The method of the invention is very simple and rapid, thus making it easy to automate by using a robot

35 apparatus where a large number of samples may be rapidly analysed. Since the preferred detection and quantification is based on a luminometric reaction, this

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can be easily followed spectrophotometrically. The use
of luminometers is well known in the art and described
in the literature.

The pyrophosphate-based nucleic acid quantification
method of the present invention thus opens up the
possibility for an automated approach for large-scale,
non-electrophoretic analysis procedures, which allow for
continuous measurement of the progress of the
polymerisation reaction with time. The method of the
invention also has the advantage that multiple samples
may be handled in parallel.

The method may be adapted for use in various
formats, for example in multi-welled microtitre plates
or micro arrays (chip arrays) etc.

The method of the present invention is particularly
advantageous in diagnosis of pathological conditions
characterised by the presence of specific DNA,
particularly latent infectious diseases such as viral
infection by herpes, hepatitis, HIV or other viruses.
Also, the method can be used with advantage to
characterise or serotype and quantify bacterial,
protozoal and fungal infections where samples of the
infecting organism maybe difficult to obtain or where an
isolated organism is difficult to grow in vitro for
subsequent characterisation as in the case of P.
falciparum or chlamydia species. Due to the simplicity
and speed of the method it may also be used to detect
other pathological agents which cause diseases such as
gonorrhoea and syphilis. Even in cases where samples of
the infecting organism may be easily obtained, the speed
of the PCR technique compared with overnight incubation
of a culture may make the method according to the
invention preferable over conventional microbiological
techniques.

The method of the present invention may be used in
the detection of specific target RNA sequences. Thus,
for example, the levels of RNA from retroviruses may be

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quantified. Alternatively, when present as a provirus,
levels of target genomic viral DNA may be quantified.
Subsequent references to viral RNA therefore include the
possibility of assessing the levels of viral DNA. The
5   method allows not only the positive identification of
samples in which the target RNA is present, but also
allow quantification of the levels of the target RNA.
This has considerable clinical utility, for example in
assessing the levels of virally infected patients over
10   time, possibly during the course of treatment to
establish the efficacy of a particular treatment or to
establish the extent of infection.

It may also be possible to use quantitative data
acquired from the method to determine the onset of viral
15   infection by extrapolation with reference to the
increasing levels of viral RNA in the same subject or
analogous subjects. This may have significant
implications in contagious diseases in which the
identification of infection onset may allow the
20   identification of other subjects which may be infected.

The method of the invention may be used for
quantifying viral RNA or DNA e.g. HIV DNA or RNA, as a
means of monitoring HIV infection. Thus viewed from a
further aspect the present invention provides a method
25   for assessing the amount of target viral (e.g. HIV) DNA
or RNA in a sample from an infected patient using the
aforementioned method.

The general method of the invention can be
performed in a number of different ways and preferred
30   embodiments of the method of the invention will now be
described.

The first of these methods involves the use of more
than one (i.e. multiple) type of competitor molecule,
preferably more than 2 e.g. 3 different competitor
35   molecules (see Fig. 1). Each competitor molecule
differs from the target and the other competitor
molecule(s) in its discriminatory sequence or equivalent

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region in the target molecule. The competitors are added
to the sample containing the target nucleic acid and co-
amplified. Preferably the primers used for
amplification are biotinylated allowing immobilisation
5   of the amplicons. The amplicons are conveniently
rendered single stranded, e.g. by magnetic bead
technology and elution. A generic extension (e.g. a
sequencing) primer is annealed substantially adjacent or
exactly adjacent to the unique discriminatory sequence
10 of the competitors and the equivalent region of the
target molecule.

      Primer extension reactions to generate sequences
complementary to part or all of the unique
discriminatory sequence of the equivalent region in the
15 target molecule can then be performed. Preferably,
sequence extension is measured indirectly, as described
above, by monitoring luminescence caused by
pyrophosphate release. The sequence extension reactions
may only involve incorporation of a single base; single
20 base extension can be achieved by incorporation of
dideoxy nucleotides which effectively "block" further
extension.

      Alternatively, and this is particularly convenient
where the discriminatory sequences comprise a
25 homopolymer of e.g. 2-6 e.g. 3 or 4 bases, two, three or
four (or more etc.) base extensions can be performed on
addition of deoxy nucleotides. For example, the deoxy
nucleotide dCTP can be added, this will only result in a
primer extension reaction with the template which
30 incorporates a homopolymeric sequence of guanine bases.
Such a template may either be derived from a competitor
of known starting concentration/copy number or from the
target nucleic acid.

      As a third alternative, a "run off" extension can
35 be performed. Here, after amplification, the sample may
be divided into a number of aliquots depending on the
number of different competitor molecules used. Then, to

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each sample dideoxy nucleotides are added, e.g. ddCTP
and ddGTP or ddATP, ddTTP and ddGTP. This "blocks" all
but one of the types of template molecule and means that
only the template whose discriminatory sequence contains
5   a base which forms a base pair with the base which has
not been added as a dideoxy nucleotide, will undergo
primer extension when deoxy nucleotides are added. Run
off extension may go beyond the discriminatory sequence
and the total signal for incorporation of e.g. 10, 20,
10   40, 80 or even 100 or 200 bases is obtained. A
different template is allowed to undergo primer
extension in each aliquot by appropriate use of dideoxy
nucleotides.

        In this way, a calibration curve can be obtained
15   from either single base extension or homopolymeric base
extension (typically of 2 to 4 bases) or run off
extension. The 'curve' which is generally substantially
linear will have multiple (i.e. 2 or more), preferably
three points derived from the signal data from the
20   competitors of known concentration/copy number. The
value of the signal obtained from primer extension of
the target templates can then be used to read off a
concentration/copy number for the amount of target
nucleic acid present in the original sample.
25   Accordingly, in one preferred embodiment, the
present invention provides a method of assessing the
amount of target nucleic acid in a sample, which
comprises
        (i)   co-amplifying the target nucleic acid together
30   with multiple competitor nucleic acid molecules, wherein
each said competitor molecule is different and comprises
a unique discriminating sequence and wherein each said
different competitor molecule is present in a different
amount;
35   (ii) performing a primer extension reaction using
each said respective amplicon obtained in step (i) as
template, using the same extension primer for each

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respective template, wherein said primer extension
reaction is template-specific; and

      (iii) determining the amounts of the respective
amplicons by detecting the results of each said primer
extension reaction; and

      (iv) assessing the amount of target nucleic acid in
the sample from said amounts.

      As mentioned above, the relative amounts of the
respective amplicons from the target and from each of
the competitor molecules may thus be determined, and
these may be correlated to provide an assessment of the
amount of target nucleic acid present in the sample.

      By "template-specific" is meant that the primer
extension reaction depends on the template, and may be
detected in a template-specific manner. In other words,
the different primer extension reactions on different
templates may be discriminated or distinguished from one
another. In this way individual or distinguishable
"signals" or results may be obtained for each of the
respective target or competitor derived templates.
Thus, the primer extension reactions may be said to be
directed, or more particularly template-directed, in the
sense that different results are obtained for different
templates. Such template-dependence may be accomplished
by using particular nucleotides for incorporation in the
primer extension step, or by restricting the
availability of the nucleotides for incorporation.

      The primer extension reactions may be performed in
different ways; as discussed above, for example by
single base extension or by multiple (i.e. 2 or more
base extensions) e.g. extension by a few bases (for
example by adding a nucleotide complementary to a
homopolymeric discriminatory region) or run-off
extension.

      These individual (i.e. separate or distinguishable)
results or signals may then be used to prepare a
calibration curve from which the amount of target may be

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determined. In the co-amplification step, the different
competitors are present in different amounts, which are
known or predetermined. Thus, a known amount (or
concentration etc.) of each competitor may be added to
5   the target nucleic acid/sample (i.e. different amounts
of the multiple competitors are co-amplified). Thus,
the primer extension reactions specific for each
amplicon will yield a different result, depending on the
amount of template present (in turn dependent upon the
10   amount of molecule originally present in or added to the
sample). In other words, the "amount" (e.g. strength or
intensity or duration) of signal may depend on the
amount of template present, and since the "signals" from
each template may be discriminated (and identified), a
15   calibration curve of signal versus amount may be
obtained. The bioluminometric PPi detection assay
discussed above is particularly suited to detecting the
primer extension reaction in this manner since it yields
quantitative information (i.e. the amount of signal is
20   dependent upon the amount of base incorporated, which in
turn is dependent upon the amount of template present).

A variation of the above method is shown in Fig. 2.
This method has the advantage that only a single species
of competitor molecule is required. According to this
25   method, multiple e.g. two or more, e.g. 3 or 4 or more,
preferably 3 calibration points can be obtained by
primer extensions of different lengths all based on the
same competitor/primer complex. A competitor molecule
of known concentration/copy number is amplified together
30   with the target nucleic acid. After amplification, the
competitor is analysed by two or more, e.g. three
parallel extension reactions, e.g. single base extension
using a dideoxy nucleotide, a homopolymeric extension of
e.g. 3-10 base pairs using deoxy nucleotides and a run
35   off extension e.g. 40-150 such as 80-120 preferably
around 100 base pairs. The sequence formed by primer
extension may include a sequence complementary to the

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discriminatory sequence or a part thereof.
Alternatively, as discussed in more detail below, the
extension primer may bind specifically to the
discriminatory sequence as a match/mismatch primer.
5   Differentiations involving match/mismatch primers is
discussed below and shown in Fig. 5. Where the
discriminatory sequence is more than one nucleotide in
length, the extension primer may hybridise to part of
the sequence and the extension reaction may involve
10   synthesis of nucleic acid which is complementary to part
of the discriminatory sequence. Thus primers will be
specific for a competitor or target amplicon but
extension will also be dependent on the bases in the
discriminatory sequence and vary between competitor and
15   target.

The datapoints can be plotted giving a linear slope
(see Fig. 3) Table 1 below gives typical values for 4
extension reactions and these values have been used in
Fig 3 wherein the y-axis corresponds to the released
20   light (i.e. amount of signal) and the x-axis corresponds
to the extension length.

Table 1

	Released light	
Extension length	Unknown	Competitor
1	5	1
3	15	3
10	50	10
100	500	100

In a similar manner (adjusting the primer used and/
or nucleotides added) the different extension are
performed on templates derived from the target. Again
35   these datapoints will generate a linear slope. The
gradient of the slopes may then be used to calculate the

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absolute amount of target. For example, if the gradient
of the slope for the competitor is 1 and this molecule
was originally present in 100 copies, if the gradient at
the slope of the target is 5 then this would have been
present in the original sample in around 500 copies.

Accordingly, in a further preferred embodiment, the
present invention provides a method of assessing the
amount of target nucleic acid in a sample, which
comprises

(i)   co-amplifying the target nucleic acid and a
known amount of a competitor nucleic acid molecule,
wherein said competitor molecule comprises a unique
discriminatory sequence, and is present in a known
amount;

(ii) performing multiple primer extension reactions
on each respective amplicon, wherein each said primer
extension reaction yields an extension product of
different length;

(iii) detecting the results of each said primer
extension reaction, on each said respective amplicon;
and

(iv) comparing the said results to determine the
relative amount of the respective amplicons in order to
provide an assessment of the amount of target nucleic
acid in the sample.

The competitor and target nucleic acid molecules,
and hence their respective amplicons, may be
distinguished by virtue of the unique discriminatory
sequence which differs in sequence from the
corresponding or equivalent sequence in the target
molecule.

The primer extension reactions on each respective
template (i.e. competitor or target-derived) may be
performed, as discussed above, using either the same
extension primer or a different primer. In other words
the extension primers may be (but need not be) specific
for either the target or competitor amplicon.

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Multiple primer extension reactions on each
amplicon are designed to yield extension products of
differing length. Such products may be distinguished
when the multiple primer extension reactions are
5   detected, and yield different detectable signals, which
may be distinguished. Thus, each primer extension
reaction will yield a separate "calibration point", and
a single competitor may thus yield multiple calibration
points, the number of which depending on the number of
10   different primer extension reactions performed. For
example, the amount of signal may vary with the length
of the extension produced. The length of extension
product may thus be plotted against signal obtained, to
generate a calibration curve. By comparing the
15   respective results obtained for the competitor and
target amplicons respectively, a correlation of the
amount of target to the amount of competitor may be
obtained. Since the amount of competitor is known, this
enables the amount of target nucleic acid in the sample
20   to be assessed.

The multiple primer extension reactions may be
designed to generate of different length as described
above e.g. single base extension, extension of a limited
number of bases (e.g. 3 to 10 or 2 to 6) for example, by
25   homopolymeric extension, or run-off extension.

A further embodiment of the present invention is
shown in Fig. 4. Here an extension primer binds
specifically to part or all of a complementary
discriminatory sequence of a competitor molecule or the
30   equivalent region in the target molecule. Again
multiple competitors of known concentrations are used
but here detection of competitor and target is
conveniently achieved using match/mismatch extension
primers. The amplification mixture is conveniently
35   immobilised and made single stranded before being
divided into aliquots, each analysed with a different
extension primer. Primers are designed with regions at

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their 3' end which are specific for just one of the discriminatory sequences (or a part thereof) or the equivalent region of the target. Thus, the extension primers hybridise specifically to either the target
5 amplicon or one of the competitor amplicons. The resulting complexes are used as substrates in primer extension assays to quantify the amount of each amplicon. The signals resulting from specific extension of each extension primer are directly correlated to the
10 original amount of the target and competitors. The competitors can thereby be used to create an internal calibration curve in order to allow estimation of the amount of target present in the sample.

    Preferably, the primer extension reaction based on
15 each template will result in the sequences of the same length to facilitate comparison. This may conveniently be achieved by designing competitor molecules which are the same as the target molecule in the region which acts as template to be growing strand in the primer extension
20 reaction. The reaction may result in incorporation of just a single nucleotide (e.g. by addition of dideoxynucleotides to the sample) or more than one nucleotide. The synthesised nucleic acid may comprise a homopolymeric sequence but as discrimination is
25 primarily achieved by match/mismatch primers, all four deoxynucleotides can be added but significant primer extension will only occur when the extension primer complementary to the discriminatory sequence or equivalent region in the target is present.
30   Accordingly, in a still further preferred embodiment, the present invention provides a method of assessing the amount of target nucleic acid in a sample, which comprises

        (i) co-amplifying the target nucleic acid together
35 with multiple competitor nucleic acid molecules, wherein each said competitor molecule is different and comprises a unique discriminating sequence and wherein each said

different competitor molecule is present in a different
amount;

(ii) performing a primer extension reaction using
each said respective amplicon obtained in step (i) as
template using a different extension primer for each
said respective template;

(iii) determining the amounts of the respective
amplicons by detecting the results of each said primer
extension reaction; and

(iv) assessing the amount of target nucleic acid in
the sample from said amounts.

Thus, different extension primers are used, each
specific for a particular template (i.e. the target
amplicon or a particular competitor amplicon).
Conveniently, as discussed above, the primers may be
designed to be specific for the unique discriminatory
region of each competitor, or for the equivalent or
corresponding region in the target. Particularly
advantageously, the primers may contain a match or
mismatch for the discriminatory region at their 3' end.
In this manner, extension will only occur where there is
a "match". By detecting the respective extension
reactions, signals may be obtained, which may be
correlated to the amount of template (and hence amount
of target/competitor) present, thereby allowing the
amount of target nucleic acid in the sample to be
assessed. In other words, as discussed for the first
preferred embodiment as described above, the relative
amounts of the respective amplicons from the target and
from each of the competitor molecules may be determined
and may be correlated to provide an assessment of the
amount of target nucleic acid in the sample.

The invention also comprises kits for carrying out
the method of the invention. These will normally
include one or more of the following components:

one or more competitor nucleic acid molecules as
defined above;

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at least one primer for the primer extension
reaction;

primer(s) for *in vitro* amplication;

nucleotides for amplication and/or for the primer
5   extension reaction (as defined above);

a polymerase enzyme for the amplification and/or
primer extension reaction; and

means for detecting primer extension (e.g. means of
detecting pyrophosphate release as discussed and defined
10   above).

Further optional components may include buffers,
etc.

The invention will now be described by way of non-
limiting examples with reference to the drawings in
15   which:-

Figure 1 shows schematically one method for the
quantification of nucleic acid using multiple DNA
competitors wherein a generic extension primer is
annealed and three different extension reactions can be
20   performed.

Figure 2 shows schematically a further method for
the quantification of nucleic acid using only a single
competitor molecule, generic extension primers and a
plurality of extension reactions.

25   Figure 3 shows a typical calibration curve derived
from the method represented in Fig. 2.

Figure 4 shows schematically a method for the
bioluminometric technique for quantification of nucleic
acid. The mixture of amplicons resulting from the
30   single-tube PCR is captured onto the solid phase and
made single-stranded, before being divided into four
aliquots and subjected to template-specific primer
extension reactions. The inorganic pyrophosphate (PPi)
released in the DNA polymerase-catalysed reaction is
35   monitored by coupled enzymatic reactions using ATP
sulfurylase and luciferase. Light generated as a result
of a successful extension is measured by a luminometer.

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Figure 5 shows HIV-1 and competitor sequences.
Nucleotides are numbered as described by Myers et al.
The outer PCR (JA79-JA82) results in a 266 bp PCR
product and the biotinylated, inner PCR product (JA80-
5   JA81) is 138 bp. The bioluminometric detection using
the three-bp 3'-end template-specific extension primers
(oligoXXX) results in extension over 110 nucleotides.

Figure 6 shows typical traces from real-time match
and mismatch primer extensions, obtained during
10 optimisation of the bioluminometric primer extension
assay. The reaction was started by addition of the
indicated deoxynucleotides. (A): Comparison between
extension primers with two and three 3'-end template-
specific bases. (B): Mismatch extensions performed with
15 0.5 and 10 pmol of extension primer, respectively. (C):
A representative example of background signal using the
final conditions (see Example for details).

Figure 7 shows luminometric traces (A) and
calibration curve (B) from bioluminometric primer
20 extension analysis of a mixture of PCR amplicons. The
plasmids pPA, pPC, pHIV-1 and pPT were amplified
separately and the PCR products were mixed in relative
amounts 1:5:25:125. The mixture was then divided into
four aliquots, each analysed with its respective
25 extension primer. The different shapes of the
bioluminescence curves reflect different enzyme kinetics
at different concentrations of extension template.

Figure 8 shows calibration curves from
bioluminometric analysis of experiments involving
30 competitive PCR. Two mixtures of plasmids were
analysed; one containing 50, 250, 1 250 and 6 250 copies
of plasmids pPA, pPC, pHIV-1 and pPT, respectively (A)
and one containing 5 000, 25 000, 125 000 and 625 000
copies (B).

35   Figure 9 shows calibration curves used for
quantification of approximately 200 (A) and 1 000 (B)
copies of MN strain HIV-1. The competitor mixture

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contained 50, 560, and 6 250 copies of plasmids pPA, pPC and pPT, respectively.

EXAMPLE 1

Preparation of MN strain HIV-1 DNA

The model system described here used DNA from HIV-1 MN infected peripheral blood mononuclear cells (PBMC). These were diluted in crude cell lysates of uninfected PBMC to contain various numbers of viral HIV-1 copies. PBMC were isolated by Ficoll-Paque density centrifugation and lysed without prior cultivation in PCR lysis buffer (10 mM Tris-HCl pH 8.3, 1 mM EDTA, 0.5% NP40, 0.5% Tween 20 and 300 mg/ml Proteinase K) at a concentration of $1x10^6$ cells/100 μl as described previously (Wahlberg et al., (1991) *AIDS Res Hum Retroviruses* 7, 983-90). Crude cell lysates were used directly for PCR amplification.

Cloning of DNA competitors

Three plasmids were constructed (pPA, pPC, pPT), containing nucleotides 244-509 of HIV-1 reverse transcriptase sequence (Myers et al., (1991) Human retroviruses and AIDS, Los Alamos National Library, Los Alamos) (Fig. 5). In these plasmids, a three or four bp substitution was introduced to enable discrimination between the wild-type HIV sequence and the three competitor sequences in the post-PCR detection system. A fourth plasmid (pHIV-1), containing wild-type HIV sequence, was also constructed. PCR cloning of competitors was achieved by mixing two partially overlapping PCR products covering the chosen region. Two ng of HIV-1 BH10 was used as template in the first reaction with JA79 and JA81 as primers, resulting in a 181 bp product. In the second reaction, resulting in a 223 bp product, the substitution primers PP1, PP2, PP3 and JA80, were each used together with JA82 to introduce

the substitutions into the three competitors and to
amplify the wild-type HIV-1 sequence. These primers are
defined in Table 2 below.

5   TABLE 2 - Oligonucleotides used

PP1	5'-GAA GAT GGA AAC CAA AAA TGA TAG GCC CAA TTG GAG G-3'
PP2	5'-GAA GAT GGA AAC CAA AAA TGA TAG GTT TAA TTG GAG G-3'
PP3	5'-GAA GAT GGA AAC CAA AAA TGA TAG GAA ATA TTG GAG G-3'
JA79	5'-ACA GGA GCA GAT GAT ACA GTA TTA G-3'
JA80	5'-GAA GAT GGA AAC CAA AAA TGA TAG G-3'
JA81	5'-biotin-CAA TTA TGT TGA CAG GTG TAG GTC C-3'
JA82	5'-CCT GGC TTT AAT TTT ACT GGT ACA G-3'
oligoAA	5'-AAC CAA AAA TGA TAG GAA-3'
oligoCC	5'-AAC CAA AAA TGA TAG GCC-3'
oligoGG	5'-AAC CAA AAA TGA TAG GGG-3'
oligoTT	5'-AAC CAA AAA TGA TAG GTT-3'
oligoAAA	5'-AAC CAA AAA TGA TAG GAA A-3'
oligoCCC	5'-AAC CAA AAA TGA TAG GCC C-3'
oligoGGG	5'-AAC CAA AAA TGA TAG GGG G-3'
oligoTTT	5'-AAC CAA AAA TGA TAG GTT T-3'
oligoCCC15	5'-AAA AAT GAT AGG CCC-3'

In the amplifications, 5 U of *Pfu* DNA polymerase
25   (Stratagene Inc, La Jolla, CA, USA) was used together
with the recommended buffer, 0.2 mM of each dNTP and 0.2
μM of each primer. The PCR program consisted of initial
denaturation at 95°C for 5 min, 30 cycles of 95°C for 30
s, 55°C for 30 s and 72°C for 2 min followed by 10 min of
30   extension at 72°C. The PCR products were separated on a
2 % agarose gel and purified using a QIAEX II kit
(Qiagen GmbH, Hilden, Germany). The purified PCR
products from the first and second reactions were mixed
individually (to generate plasmids pPA, pPC, pHIV-1 and
35   pPT) and used as template in a third PCR with the
primers JA79 and JA82. AmpliTaq Gold DNA polymerase
(Perkin-Elmer, Norwalk, CT, USA) was used with the

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recommended buffer and 2 mM MgCl$_2$, 0.2 mM of each dNTP
and 0.2 μM of each primer, which were added in the first
PCR cycle.

The PCR program was as follows: 95°C for 12 min,
68°C for 12 min, 25 cycles of 95°C for 30 s, 60°C for 30
s, 72°C for 1 min and finally 10 min of extension at
72°C. The four 266 bp products were purified using QIAEX
II (Qiagen), ligated into AT cloning vector pGEM-T
(Promega, Madison, WI, USA) and transformed into
Escherichia coli RR1ΔM15 (Ruther, U. (1982) *Nucleic
Acids Res* **10**, 5765-72). The resulting clones were PCR
screened and sequenced (Hultman et al., (1989) *Nucleic
Acids Res* **17**, 4937-46). Correct clones of the four
constructs were purified using Jetstar plasmid
preparation kit (Genomed Inc., NC, USA). Plasmids were
dissolved in 10 mM Tris-HCl (pH 8.3) and 2 mM EDTA and
were analysed by agarose gel electrophoresis and by
measurement of absorbance.

End-point dilution analysis

Determination of plasmid concentrations was
performed by using end-point dilution experiments
involving nested PCR as outlined previously (Vener et
al., (1996) *Biotechniques* **21**, 248-52, 253-5). The
plasmid preparations were diluted in PCR buffer II
(Perkin Elmer) containing 10 ng/μl yeast RNA (Boehringer
Mannheim, Mannheim, Germany). The numbers of DNA copies
were calculated by the Poisson distribution formula (*i.
e.* one starting copy corresponds to a dilution step in
which 63% of the samples are positive by PCR (Brinchmann
et al., (1991) *J Virol* **65**, 2019-23)).

Competitive PCR

HIV-1 target and competitors were amplified with
nested primers (see below) located in the polymerase
gene of the HIV-1 genome. For the initial experiments,
1x10^6 copies of the four plasmid constructs (containing

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the HIV wild-type sequence and the three competitor
sequences, respectively) were amplified separately.
During optimisation of the bioluminometric detection
system, these PCR products were used pure or as a
mixture of the four PCR products. In the experiments
including competitive PCR, different configurations of
plasmids were used as template. Mix A (50, 250, 1 250
and 6 250 copies of plasmids pPA, pPC, pHIV-1 and pPT
respectively) and mix B (5 000, 25 000, 125 000 and 625
000 copies) were used to investigate whether the four
plasmids were PCR amplified with the same efficiency.
Mix C (50, 560 and 6 250 copies) was used for
quantification of HIV-1 DNA.

      The nested PCR was performed as follows: The
templates (four plasmids or HIV-1 MN infected PBMC mixed
with three competitors) were added to the outer PCR
mixture resulting in final concentrations of 0.1 μM of
each primer (JA 79 and JA 82), 50 μM of each dNTP, 1 U
of AmpliTaq Gold DNA Polymerase, 10 mM Tris-HCl (pH
8.3), 50 mM KCl and 2.5 mM $MgCl_2$ in a reaction volume of
50 μl. The PCR program consisted of initial
denaturation at 96°C for 10 min, followed by a 30-cycle
program consisting of 92°C for 30 s, 50°C for 30 s and
72°C for 30 s, followed by extension at 72°C for 1 min.
In the inner amplification (primers JA80 and JA81), 2.5
μl of outer PCR product was used as template. The
amplification conditions were the same as in the outer
PCR except that 35 cycles of amplification was used.

Bioluminometric quantification of PCR products

      To prepare the template for quantification, 5 μl of
biotinylated PCR products were immobilised onto 300 μg
streptavidin-coated super paramagnetic beads (Dynal AS,
Oslo, Norway) as described by the manufacturer. Single-
stranded DNA was obtained by removing the supernatant
after incubation with 0.1 M NaOH for 5 min. The
immobilised single-stranded DNA was washed once with 10

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mM Tris-HCl (pH 7.5). Extension primers (0.5 pmol)
oligoAAA, oligoCCC, oligoGGG and oligoTTT (Table 2) were
then hybridised to the single-stranded DNA in 20 µl of
buffer containing 0.1 M Tris-acetate (pH 7.75) and 20 mM
5   MgAc$_2$ at 50°C for 5 min after heating of the mixture at
95°C for 1 min. The primed immobilised PCR products
were washed once with binding/washing buffer (10 mM
Tris-HCl (pH 7.5), 2 M NaCl, 1 mM EDTA, 0.1% Tween 20)
and once with 0.1 M Tris-acetate (pH 7.75) buffer before
10   addition of the 200 µl standard assay volume containing
0.1 M Tris-acetate (pH 7.75), 0.5 mM EDTA, 5 mM MgAc$_2$,
0.1% bovine serum albumin, 1 mM dithiothreitol, 5 µM
adenosine 5'-phosphosulfate (APS), 80 µg
polyvinylpyrrolidone (360 000), 20 µg D-luciferin
15   (BioOrbit, Finland), 4 U of an exonuclease-deficient T7
DNA polymerase (Sequenase 2.0; US Biochemical,
Cleveland, OH, USA), 30 mU of ATP sulfurylase
(ATP:sulfate adenylyl transferase; EC 2.7.7.4) (Sigma
Chemical Co., St Louis, MO, USA), 40 ng of luciferase
20   (Biothema, Sweden).

        The reaction was started by the addition of 400
pmol of each dNTP, where deoxyadenosine-thiotriphosphate
(dATPαS) and deoxythymidine-thiotriphosphate (dTTPαS)
were substituted for the natural deoxynucleotides dATP
25   and dTTP (Nyrén, P., Karamohamed et al., (1997) *Anal
Biochem* **244,** 367-73). The reaction was carried out at
room temperature. The PPi released due to nucleotide
incorporation was detected by the above-described system
using a luminometer and a potentiometric recorder. The
30   signal was measured 1 min after the reaction was
started. The luminescence output was calibrated by the
addition of a known amount of inorganic pyrophosphate
(PPi).

35   **Optimisation of the bioluminometric primer extension
assay**
        During the initial optimisation experiments,

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template-specific extension primers differing by two
nucleotides at their 3'-termini (oligoAA, oligoCC,
oligoGG and oligoTT (Table 2)) were used. A shorter
primer with three 3' template-specific nucleotides
5   (oligoCCC15 (Table 2)) was also synthesised and compared
to the nineteen-bp primer oligoCCC. The amount of
extension primer was initially 10 pmol, but was in later
experiments reduced to 0.5 pmol. Different combinations
of the natural deoxynucleotides and α-thiotriphosphate
10   deoxynucleosides were used in the primer extension assay
to achieve a low background signal and a high
polymerisation rate. Under suboptimal conditions, the
reaction rate was slower and therefore the signal
intensity was measured after more than 1 min (i.e. when
15   the match signal had reached the plateau phase).

RESULTS

Principle of the quantification method

        The principle of the competitive quantification
20   method described above is outlined in Figure 4. In this
model system, the target (HIV-1 DNA) is co-amplified
using nested primers with three plasmid DNA competitors
designed to differ at only three or four adjacent
nucleotide positions. The resulting biotinylated PCR
25   fragments are immobilised onto streptavidin-coated
paramagnetic beads. After alkali treatment, immobilised
single-stranded DNA is divided into four aliquots, which
are each mixed with one of four specific
oligonucleotides. These extension primers hybridise
30   specifically to either the wild-type HIV amplicon or to
one of the three competitor amplicons. The four
resulting complexes are used as substrates in separate
primer extension assays to quantify the amount of each
amplicon. The signals resulting from specific extension
35   of each extension primer are directly correlated to the
original amount of the target and competitors. The
three competitors can thereby be used to create an

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internal calibration curve in order to allow estimation
of the HIV target.

Construction of DNA competitors

5   Three plasmid competitors (pPA, pPC and pPT) were
designed and constructed by PCR cloning to contain
mutated HIV-1 polymerase sequence (Fig. 5). PCR
amplification of wild-type HIV-1 and the three
competitors results in four amplicons of the same length

10   (138 bp), but differing at the three or four nucleotides
immediately downstream inner PCR primer JA80. The
alterations were designed in a 3 bp homopolymeric
stretch, i.e. the wild-type sequence comprising of three
adjacent G nucleotides. The four mutated nucleotides

15   enable discrimination between the amplicons using four
specific extension primers in the primer extension
assay. A plasmid containing HIV wild-type sequence
(pHIV-1) was also constructed, to be used as target in
the model system. The plasmid concentrations were

20   determined by using limiting dilution analysis
(Brinchmann et al., (1991) *J Virol* **65**, 2019-23.).
Mixtures of known amounts of the three competitors were
then co-amplified with the wild-type HIV-1 target in a
nested PCR.

25

**Optimisation of the bioluminometric primer extension
assay**

<u>Design and specificity of extension primers</u>

30   In order to evaluate the specificity of primer
extension on the different constructed competitors,
several "run-off" extension experiments were performed.
The individual plasmid constructs were amplified and
immobilised onto streptavidin-coated paramagnetic beads

35   and rendered single-stranded by NaOH. Initially, these
templates were investigated with four extension primers
(oligoAA, oligoCC, oligoGG and oligoTT) designed to have

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two nucleotides at their 3'-ends complementary to only their respective target. When extension was performed on all combinations of extension primer and template, high background levels were obtained for some of the

5   mismatch extensions. The combination resulting in the highest level of background was a two T:C (primer:template) mismatch (approximately 12% for extension primer oligoTT hybridised to competitor pHIV-1 (data not shown)).

10   Since the extension primer oligoTT was responsible for the highest level of background on all tested templates, an oligonucleotide with three template-specific bases at the 3'-end was synthesised (oligoTTT). When the new primer (oligoTTT) was compared to oligoTT

15   in an experiment involving match and mismatch extensions, the background levels were reduced to approximately 13-37% (depending on target) of the background signals obtained with oligoTT. Figure 6A shows the unspecific extensions by primers with two and

20   three mismatches at the 3'-end on the template pHIV-1, for which the largest decrease in mismatch signal was observed. The arrow indicates the point of nucleotide addition and the accenting curve indicates the polymerase activity (in pmol of PPi produced) and time

25   elapsed is indicated. In the assay, released pyrophosphate (PPi) is converted to ATP by ATP sulfurylase and the sequential production of light from ATP by luciferase is detected by a luminometer. Since dATP is a false substrate for luciferase, it was

30   replaced by dATPαS which is silent for luciferase and efficiently incorporated by the DNA polymerase (Ronaghi M. et al (1996) Anal. Biochem 242, 84-9).

Use of nucleotide analogs

35   Another effort to reduce the mismatch extensions was to substitute the α-thiotriphosphate analogs (αS-dNTPs) for the remaining deoxynucleotides, since earlier

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studies have shown an improved discrimination between
match and mismatch signals (one-bp mismatches (Nyrén et
al., (1997) *Anal Biochem* **244**, 367-73)). For some
primer:template mismatch combinations (T:G and C:A) we
5   observed a dramatic decrease in background, while the
substitution was less effective in reducing the
previously described background in extension of oligoTT
hybridised to pHIV-1 (T:C mismatch). A drawback using
α-thiotriphosphate analogs is the slower polymerisation
10   rate in the match extensions, therefore, only the first
correct bases after the 3'-mismatch termini (dATP and
dTTP, see Fig. 5) were substituted and used together
with the natural dCTP and dGTP in the final competitive
PCR experiments.
15

Concentrations of extension primer
        When performing extensions on all match and
mismatch combinations using three-bp 3' specific
extension primers (oligoAAA, oligoCCC, oligoGGG and
20   oligoTTT), oligoCCC hybridised to pHIV-1 (a C:C
(primer:template) mismatch) surprisingly resulted in a
background of approximately 11%, which was much higher
than in the initial experiments, using the two-bases 3'
specific primer oligoCC. Since we believed that this
25   background could be caused by mispriming rather than 3'
mismatch extension, the amount of extension primer was
reduced from 10 to 0.5 pmol. This resulted in a
background comparable to the other mismatch combinations
(approximately 4% of the match signal) without affecting
30   the corresponding match signal (Fig. 6B).
        A four bases shorter primer (oligoCCC15) was
synthesised and hybridised to pHIV-1 (0.5 and 10 pmol of
primer, respectively). In the extension reactions where
10 pmol of primer was used in the hybridisation step,
35   the shorter primer resulted in a considerably lower
background, but for 0.5 pmol of primer no significant
difference was observed.

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Dynamic range of the bioluminometric primer extension assay

The optimised protocol, in respect to mismatch extension and reaction rate was thus composed of the
5   four extension primers with three alternating nucleotide stretches at their 3'-ends (oligoAAA, oligoCCC, oligoGGG and oligoTTT), specific for HIV-1 and the three competitors. To further increase the difference of primer extension rate of a match over a mismatch primer,
10 the α-thio triphosphate analog for the first correct deoxynucleotide to be incorporated (dATP and dTTP) were used. All combinations of extension primer and PCR amplicon were analysed and all background signals were approximately 5% of the corresponding match signals. An
15 example of the optimised system in which extension of competitor pPC amplicon hybridised to its corresponding extension primer oligoCCC is shown in Fig. 6 C. The background signal from the combination of competitor pPC and extension primer oligoTTT i.e. a T:G
20 (primer:template) mismatch is also demonstrated.

To further investigate the detection limit and the linearity of the bioluminometric primer extension assay, various amounts of one competitor amplicon (pPA) were detected in a background consisting of a mixture of the
25 other three amplicons (pPC, pHIV-1 and pPT). It was possible to reproducibly detect 0.025 μl of pPA PCR product in a total of 5 μl PCR product, corresponding to a sensitivity of 1:200 (data not shown). In addition, a mixture of all four PCR amplicons (pPA, pPC, pHIV-1 and
30 pPT in approximative relative amounts 1:5:25:125) was prepared, divided into four aliquots and analysed separately with all four extension primers. The obtained results demonstrate a linearity and conformity between duplicate samples (Fig. 7A and B). The four
35 different amplicons were also mixed in another configuration (125:25:5:1) and similar results were obtained (data not shown).

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Competitive PCR on mixtures of four plasmids

An important goal of the developed system was to allow for a single tube co-amplification of the multiple competitors requiring similar amplification efficiencies for the competitors and the target. Prior to co-amplification, the individual plasmid constructs were quantified by end-dilution series and nested PCR that give a statistical estimation of plasmid copy number (27, 35). Two different mixtures of all four plasmid templates were prepared; one low-copy number mixture containing 50, 250, 1 250 and 6 250 copies (mix A) and one high-copy number mixture containing 5 000, 25 000, 125 000 and 625 000 copies (mix B) of plasmids pPA, pPC, pPG and pPT, respectively. After nested PCR, the products were analysed by using the bioluminometric primer extension method. The results for the low-copy and high-copy mixtures (Fig. 8A and B) show linearity in both intervals, thus indicating similar amplification efficiencies.

Quantification of HIV-1 MN strain

To evaluate the quantification method on proviral HIV-1 DNA, a new competitor mixture containing 50, 560 and 6 250 copies (mix C) of plasmids pPA, pPC and pPT respectively was established. This mixture was then used with 200 and 1000 copies of HIV-1 MN DNA, also estimated by separate end-dilution experiments. Competitive PCR was followed by bioluminometric analysis as outlined above. The corresponding curves are shown in Fig. 9A and B. A linear response was obtained making an estimation of the target copy number possible by using the internal calibration curves, which give an estimation of 203 and 1072 MN copies, respectively. The obtained results are in good agreement with the results obtained from end-point experiments.

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Claims

1.   A method of assessing the amount of target nucleic
acid in a sample which comprises co-amplifying the
target nucleic acid and at least one competitor nucleic
acid molecule wherein each competitor molecule contains
a unique discriminatory sequence, and determining the
relative amounts of the respective amplicons,
characterised in that determination is achieved by
detecting a primer extension reaction using each said
amplicon as template.

2.   A method as claimed in claim 1, said method
comprising correlating the relative amounts of the
respective amplicons to assess the amount of target
nucleic acid in the sample.

3.   A method as claimed in claim 1 or claim 2, wherein
said competitor molecule is identical to said target
nucleic acid except in the region of the unique
discriminatory sequence.

4.   A method as claimed in any one of claims 1 to 3,
wherein said discriminatory sequence comprises 1 to 10
bases.

5.   A method as claimed in any one of claims 1 to 4,
wherein said discriminatory sequence comprises 3 to 5
bases.

6.   A method as claimed in any one of claims 1 to 5,
wherein the discriminatory sequence is a homopolymeric
region.

7.   A method as claimed in any one of claims 1 to 6,
wherein the primer extension reactions are performed
using the same primer for the target- and competitor-

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derived amplicons.

8.   A method as claimed in any one of claims 1 to 6,
wherein the primer extension reactions are performed
using a different primer for the target- and competitor-
derived amplicons.

9.   A method as claimed in any one of claims 1 to 8,
wherein the primer extension reactions are detected by
detecting pyrophosphate (ppi) release.

10.   A method as claimed in claim 9, wherein
pyrophosphate (ppi) is detected luminometrically.

11.   A method as claimed in claim 10, wherein
pyrophosphate is detected enzymically using the enzyme
luciferase as a ppi-detection enzyme.

12.   A method as claimed in claim 11, wherein in the
primer extension reaction, an α-thio analogue of an
adenine nucleotide is used.

13.   A method as claimed in any one of claims 1 to 12,
wherein said target and competitor nucleic acid
molecules are co-amplified using amplification primers
which are immobilised or carry means for immobilisation.

14.   A method as claimed in any one of claims 1 to 13,
wherein multiple competitor molecules are used.

15.   A method as claimed in claim 14, wherein 2 to 6
competitor molecules are used.

16.   A method as claimed in claim 14 and claim 15
wherein different amounts of each said competitor
molecule are used.

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17. A method as claimed in any one of claims 14 to 16
wherein primer extension reactions are performed on said
respective amplicons using the same extension primer,
wherein each said primer extension reaction is template-
5   specific.

18. A method as claimed in any one of claims 14 to 16
wherein primer extension reactions are performed on said
respective amplicons using a different template-specific
10   extension primer on each said template.

19. A method as claimed in any one of claims 1 to 13,
wherein a single competitor molecule is used.

15   20. A method as claimed in claim 19, wherein multiple
primer extension reactions are performed on each said
respective target- or competitor derived amplicon,
wherein each said primer extension reaction yields an
extension product of different length.
20

21. A method as claimed in claim 20, wherein 2 to 6
primer extension reactions are performed.

22. A kit for use in a method as defined in any one of
25   claims 1 to 21, said kit comprising
     (a) at least one competitor molecule as defined in
any one of claims 1 to 21; and
     (b) means for detecting a primer extension
reaction.
30

23. A kit as claimed in claim 22, further comprising
one or more of the following components:
     (c) at least one extension primer;
     (d) primer(s) for *in vitro* amplication;
35   (e) a polymerase enzyme for the amplification
and/or primer extension reaction.
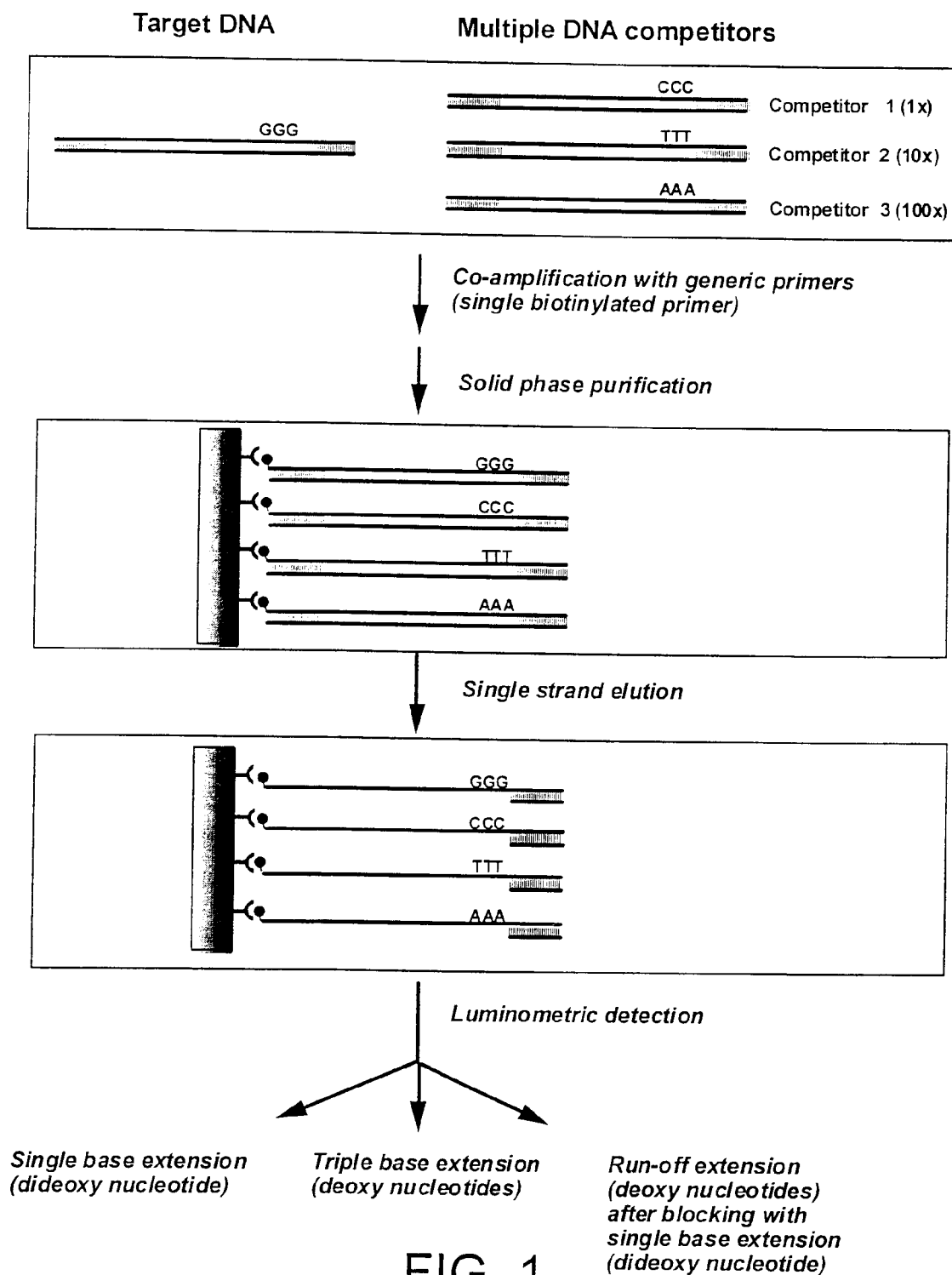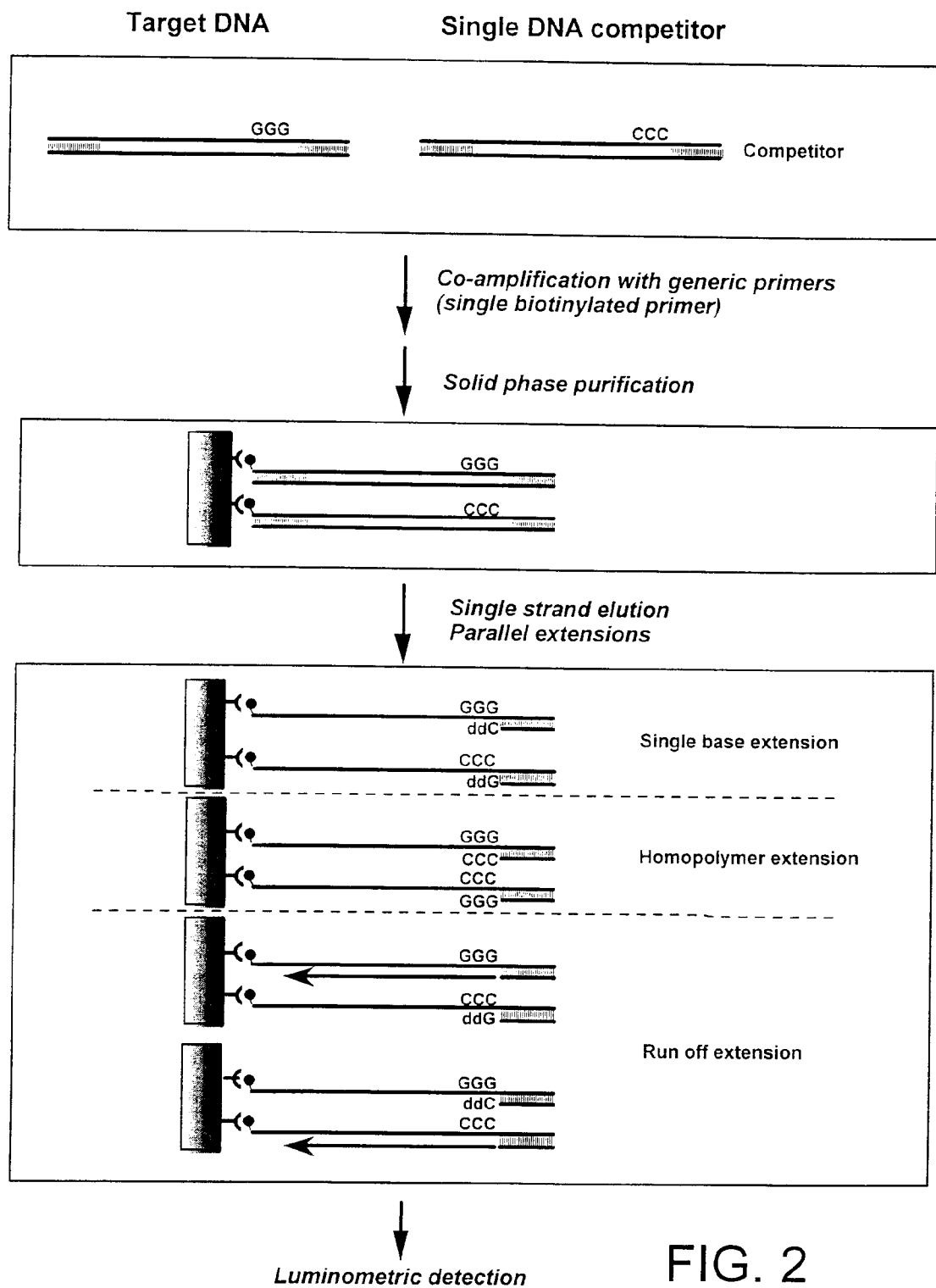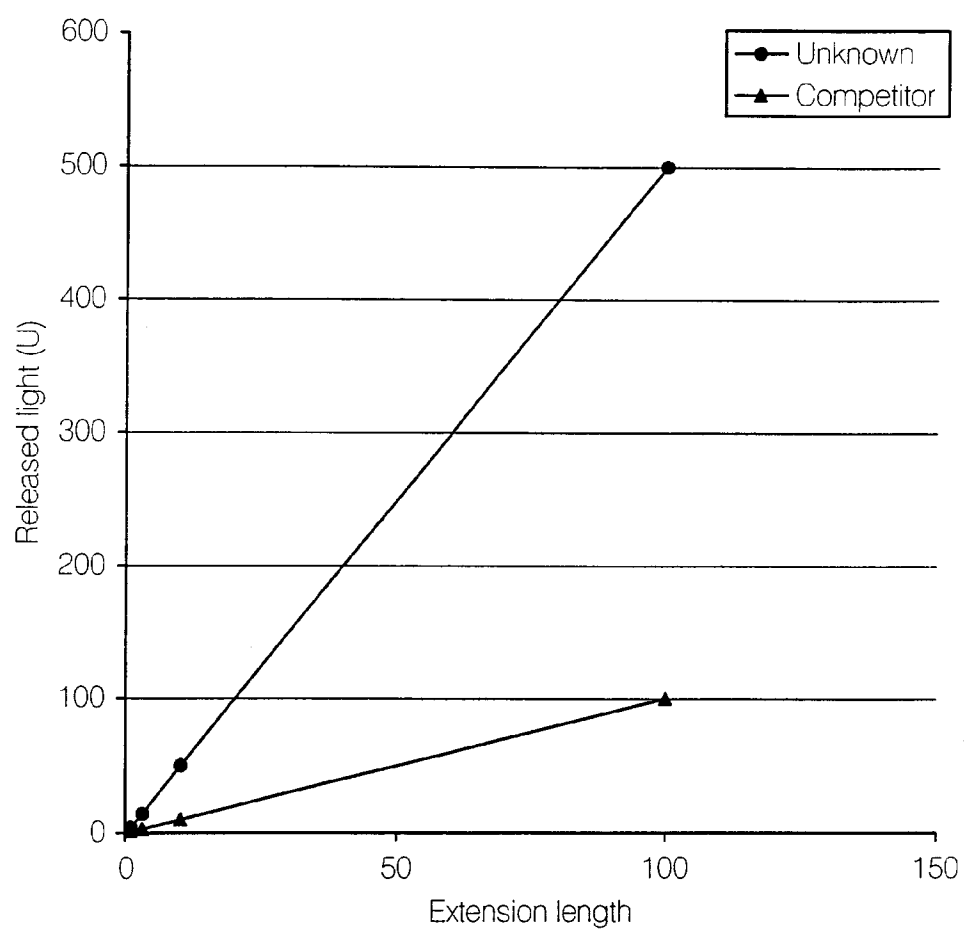
Target DNA　　　　　**Multiple DNA competitors**

Co-amplification with generic primers
(single biotinylated primer)

Solid phase purification

Single strand elution

Luminometric detection

Single base extension
(dideoxy nucleotide)

Triple base extension
(deoxy nucleotides)

Run-off extension
(deoxy nucleotides)
after blocking with
single base extension
(dideoxy nucleotide)

FIG. 1

Target DNA   Single DNA competitor

FIG. 2

Luminometric detection

FIG. 3

Quantification using single competitor
and multiple extensions

Target DNA   **Multiple DNA competitors**

Co-amplification with generic primers
(single biotinylated primer)

Solid-phase purification

Single-strand elution and
separate primer hybridisation

primer extension

Bioluminometric detection

FIG. 4

FIG. 5

FIG. 6a

pHIV-1
oligoGGG
(0.5 pmol)

↑ 1 pmol

⊢►⊣
1 min

pHIV-1
oligoCCC
(10 pmol)

pHIV-1
oligoCCC
(0.5 pmol)

↑   ↑   ↑

dATPαS, dCTP,   dATPαS, dCTP,   dATPαS, dCTP,
dGTP, dTTP   dGTP, dTTP   dGTP, dTTP
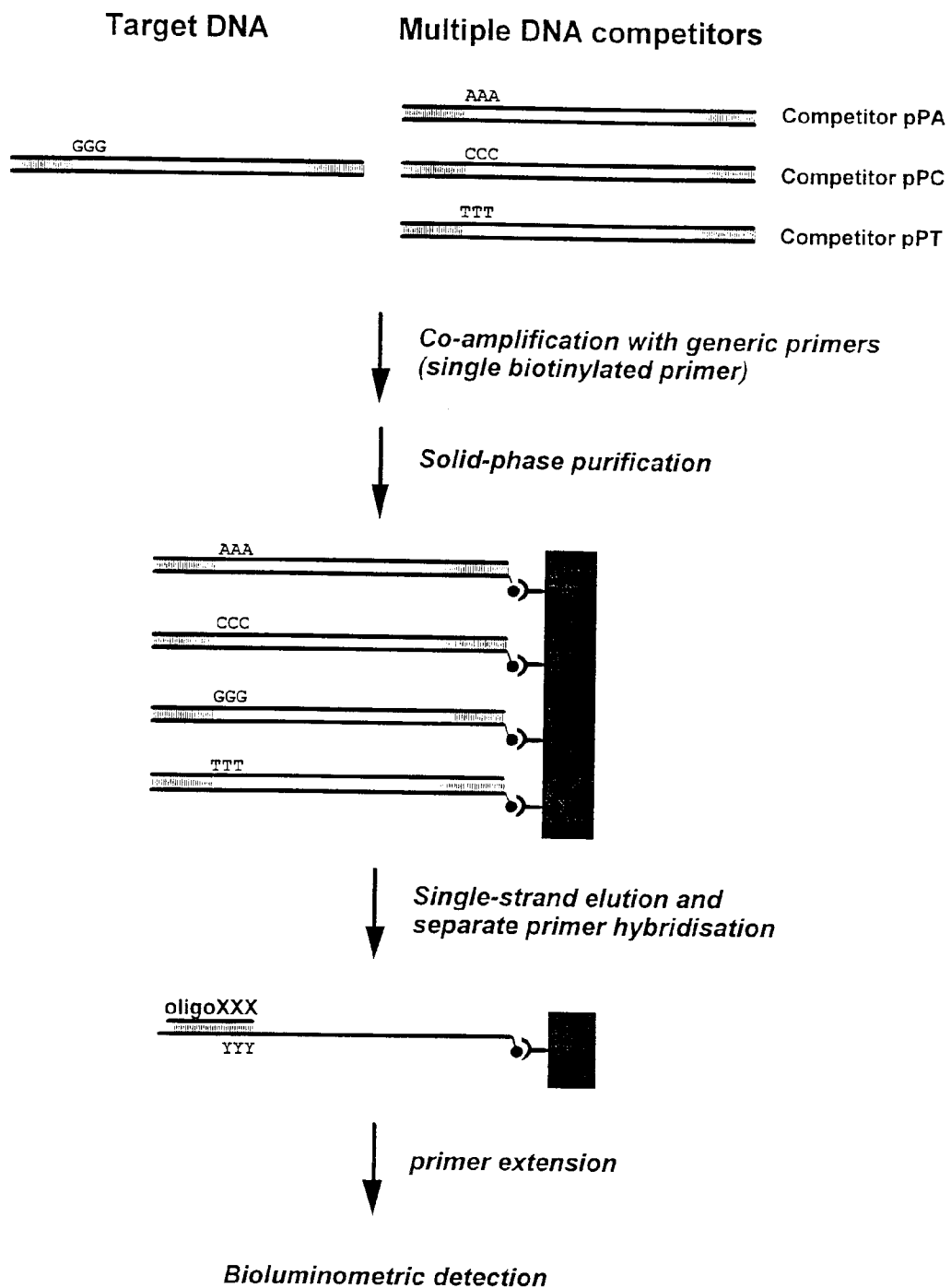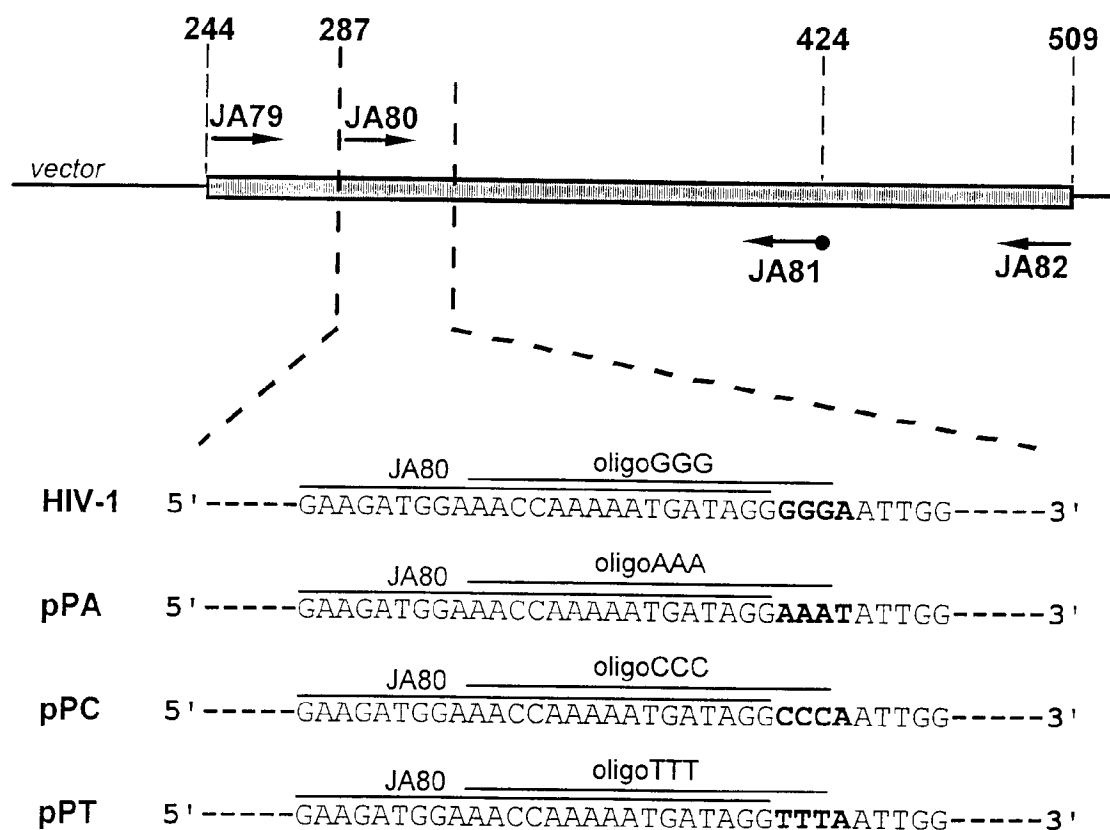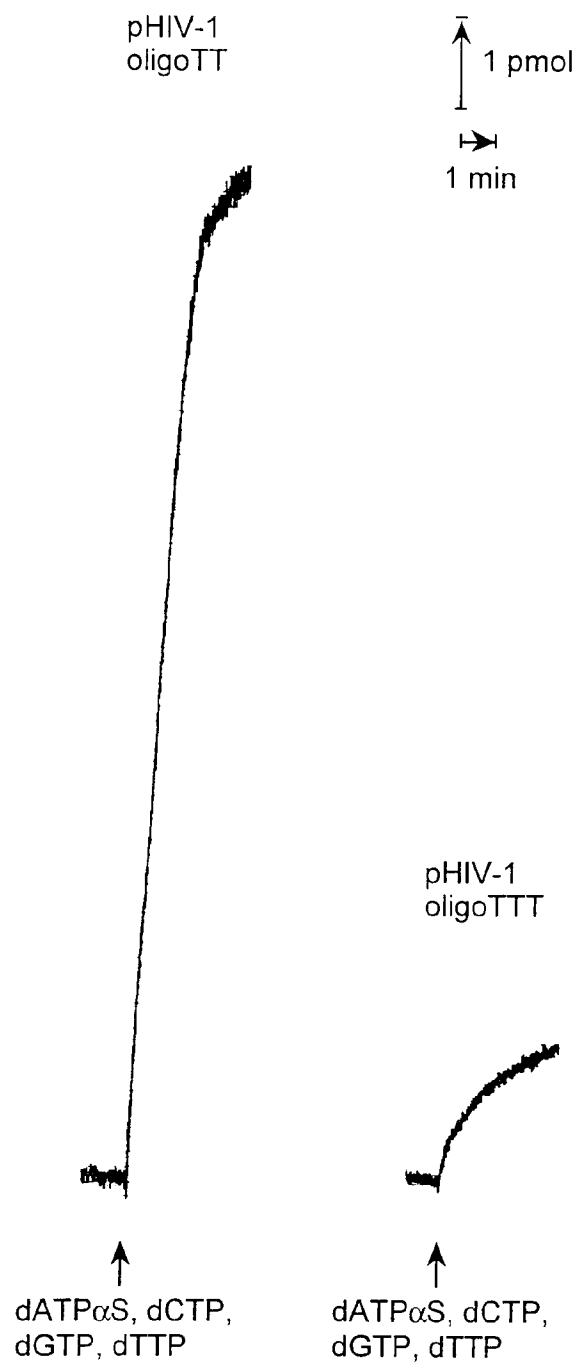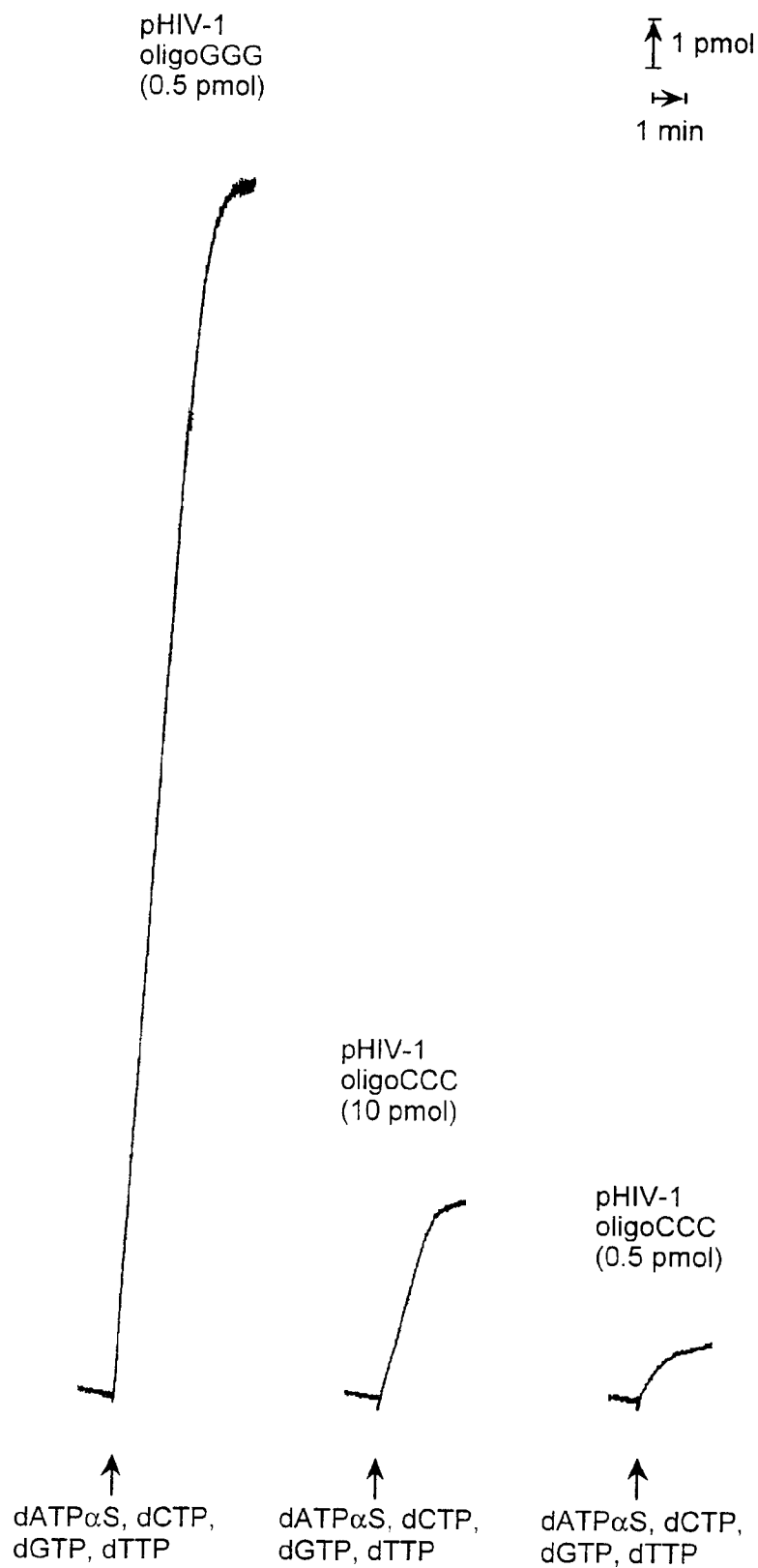
FIG. 6b

SUBSTITUTE SHEET (RULE 26)

FIG. 6c

FIG. 7a

FIG. 7b

y = 5.2564 + 4.9070e-3x   R^2 = 1.000

FIG. 8a

y = 4.9908 + 8.6201e-5x   R^2 = 1.000

FIG. 8b

FIG. 9a

FIG. 9b